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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : **C12N 15/75, 15/65**	**A1**	(11) International Publication Number: **WO 96/23073**
		(43) International Publication Date: 1 August 1996 (01.08.96)

(21) International Application Number: PCT/DK96/00038

(22) International Filing Date: 23 January 1996 (23.01.96)

(30) Priority Data:
0083/95   23 January 1995 (23.01.95)   DK
0799/95   6 July 1995 (06.07.95)   DK

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(81) Designated States: AL, AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

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(54) Title: DNA INTEGRATION BY TRANSPOSITION

(57) Abstract

Multicopy strains of gram-positive bacteria carrying multiple copies of a DNA sequence of interest may be constructed by use of a method involving introduction of a DNA construct comprising the DNA sequence of interest into the genome of the recipient cell by transposition and subsequent deletion of a marker gene used for selection of cells having received the DNA construct by a resolution system. The multicopy strains are preferably free from a gene encoding an undesirable marker such as an antibiotic resistance marker.

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DNA INTEGRATION BY TRANSPOSITION

FIELD OF THE INVENTION

The present invention relates to a novel DNA construct useful
for the construction of a bacterial cell having integrated more
5 than one copy of a DNA sequence of interest into its genome,
which cell may be free of any selection markers, and a method
of constructing such cells.

BACKGROUND OF THE INVENTION

Prokaryotic transposable elements are discrete DNA sequences
10 capable of insertion at single or multiple sites within a
prokaryotic genome. Normally, such elements consist of a gene
encoding a transposase protein and a transposable cassette
comprising a resistance gene flanked by sequences recognized by
the transposase protein. Transposition of the transposable
15 cassette into the genome of a host cell (which may, e.g. take
place at random or at hot spot sites) occurs via recognition
and interaction with the flanking sequences of the transposable
cassette by the transposase protein.

Different classes of transposable elements exist. One class
20 comprises i) insertion sequences (IS) which are small (less
than 2 kb) DNA fragments encoding transposase proteins or other
determinants mediating transposition, and ii) composite
transposons, i.e. DNA fragments flanked by two copies of an
insertion sequence. The terminal portions of all IS sequences
25 comprises inverted repeat sequences. The transposase protein
functions by recognizing these terminal sequences and interac-
ting with the sequences to effect transpositions within the
genome.

The second class of transposons is the Tn3 family of tranpo-
30 sons. These transposons encode two products involved in a two-
step transposition process: a transposase and a resolvase.

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Transposons belonging to this second class have inverted terminal repeats of approximately 35-40 bp.

The third class includes bacteriophage Mu and related phages. Bacteriophage Mu is large relative to other transposons with a
5 genome of 36 kb. Mu encodes two gene products which are involved in the transposition process, a 70 kDa transposase and an accessory protein of approximately 33 kDa. An unusual feature of Mu that distinguishes it from other tranposons is that its ends are not inverted repeat sequences. The Mu
10 transposase has, however, been shown to bind to both ends in an in vitro binding assay.

Transposons have been used extensively for mutagenesis and cloning in gram-positive and gram-negative bacteria: Youngman, P. J., Perkins, J. B., Losick, R. (1983) Genetic transposition
15 and insertional mutagenesis in Bacillus subtilis with Streptococcus faecalis transposon Tn917, Proc. Natl. Acad. Sci. USA, 80, 2305-2309; Youngman, P., Perkins, J.B., Losick, R. (1984), Construction of a cloning site near one end of Tn917 into which foreign DNA may be inserted without affecting transposition in
20 Bacillus subtilis or expression of the transposon-borne erm gene, Plasmid 12, 1-9; Youngman, P. (1985) Plasmid vectors for recovering and expoliting Tn917 transpositions in Bacillus subtilis and other gram positives, p. 79-103 in K. Hardy (ed.), Plasmids: a practical approach, IRL Press, Oxford; Kleckner,
25 N., Roth, J., Botstein, D. (1977) Genetic engineering in vivo using translocatable drug-resistance elements. New methods in bacterial genetics, J. Mol. Biol., 116, 125-159; Wati, M. R., Priest, F. G., Mitchell, W. J. (1990) Mutagenesis using Tn917 in Bacillus licheniformis. FEMS microbiol. Lett., 71, 211-214;
30 Petit, M.-A., Bruand, C., Janniere, L., Ehrlich, S. D. (1990) Tn10-derived transposons active in Bacillus subtilis. J. Bacteriol., 172, 6736-6740.

The latter reference describes pHV1248 and pHV1249, plasmids that are thermosensitive for replication, which carry a

transposase gene from Tn10 modified to be expressed in <u>B.</u>
<u>subtilis</u>, and sufficient sequences from the IS10 elements of
Tn10 flanking a chloramphenicol resistance gene (mini-Tn10) to
allow transposition of mini-Tn10 into the <u>B. subtilis</u> chromoso-
5 me.

Maguin et al. (Maguin, E., Duwat, P., Hege, T., Ehrlich, D,
Gruss, A. (1992) New thermosensitive plasmid for gram-positive
bacteria, J. Bacteriol. 174, 5633-5638) describe an alternative
version of this system.

10 EP 485 701 discloses the use of transposons for introduction of
single copies of a DNA sequence into a prokaryotic cell genome,
the transposase protein being encoded in cis.

Slugenova et al., ((1993), Enhanced α-amylase production by
chromosomal integrtation of pTVA1 in industrial strain in <u>B.</u>
15 <u>subtilis</u>, Biotechnology Letters, 15, 483-488) describe the
multiple integration of plasmid pTVA1 comprising a modified
transposon Tn917 and an α-amylase gene of interest located
outside the transposon. Antibiotic resistance marker genes are
present in the resulting strain.

20 EP 0 332 488 describes a transposition based system for
construction of multicopy bacterial strains, i.e. strains
comprising multiple copies of a gene of interest, which strains
further comprise multiple copies of a selectable marker gene
introduced with the gene of interest. The system is exemplified
25 by use of a phage Mu transposon for modification of gram-
negative bacteria.

WO 95/01095 describes the use of a minitransposon as a vector
for stably tranforming an exogenous gene into a eukaryotic
(e.g. animal) chromosome.

30 Simon, R., Priefer, U., Pühler, A. ((1983), A broad host range
mobilization system for in vivo genetic enginering: Transposon

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mutagenesis in gram-negative bacteria, Bio/Technology, 1, 784-
791) describe the use of E. coli specific vectors to transfer
transposons into other gram-negative strains by conjugation.

Several of the above described multicopy strains have been
5 produced by integration of a genetic construct comprising the
gene of interest and an antibiotic selectable marker and
amplifying said construct by culturing the cell in the presence
of increasing dosages of antibiotic. Thus, the resulting cell
typically comprises a number of antibiotic resistance genes.
10 The presence of such genes are undesirable, in particular from
an environmental and a product approval point of view.

Non-antibiotic selection markers have been used for construc-
tion of multicopy strains. Herrero, M., de Lorenzo, V., Timmis,
K.N. ((1990), Transposon vectors containing non-antibiotic
15 resistance selection markers for cloning and stable chromosomal
insertion of foreign genes in gram-negative bacteria, J.
Bacteriol., 172, 6557-6567) describe such as system in which
herbicide or heavy metal resistances are used as selection
markers.

20 Another alternative to the use of antibiotic resistance markers
are described in DE 4 231 764 in which an alternating selection
of Thy⁻ (trimethoprim resistance) and Thy⁺ (thy prototrophy) is
used for introduction of product genes in Bacillus spp. thereby
avoiding the need for selectable markers.

25 Specific deletion of DNA segments from the chromosomes of bac-
terial species have traditionally been performed by the methods
of homologous recombination (Hamilton, C. H., Aldea, M., Wash-
burn, B. K., Babitzke, P. (1989). New method of generating
deletions and gene replacement in Escherichia coli. J. Bac-
30 teriol., 171, 4617-4622.; Maguin, E., Duwat, P., Hege, T.,
Ehrlich, D, Gruss, A. (1992). New thermosensitive plasmid for
gram-positive bacteria. J. Bacteriol. 174, 5633-5638.).
However, the use of homologous recombination to delete re-

sistance marker genes from strains having multiple, tandem copies of such genes each linked to a copy of the gene of interest is hardly applicable, as homologous recombination would delete also the extra copies of the gene of interest.

5 The concept of using site specific recombination systems for integration and retrieval of sequences from the bacterial chromosome, using elements from either phage lambda or P1, and some specific methods of achieving this, has been described (Hasan, N., Koob, M., Szybalski, W. (1994), Escherichia coli 10 genome targeting, I. Cre-lox-mediated in vitro generation of ori⁻ plasmids and their in vivo chromosomal integration and retrieval. Gene, 150, 51-56). The cre-lox system is further described by Abremski, K., Hoess, R., Sternberg, N. (1983), Studies on the properties of P1 site-specific recombination: 15 Evidence for topologically unlinked products following recombination, Cell, 32, 1301-1311. An alternative system is based on the recombination system of the broad-host range plasmid RP4 (Eberl, L., Kristensen, C.S., Givskov, M., Grohmann, E., Gerlitz, M., Schwab, H. (1994), Analysis of the multimer 20 resolution system encoded by the parCBA operon of broad-host-range plasmid RP4, Mol. Microbiol., 12, 131-141)). Stark, W.M., Boocock, M.R., Sherratt, D.J. (1992), Catalysis by site-specific recombinases, Trends in Genetics, 8, 432-439) is a review article on the mechanism of resolvase action. Camilli et 25 al. ((1994), Use of genetic recombination as a reporter of gene expression, Proc. Natl. Acad. Sci. USA, 91, 2634-2638) describe the use of res sites and resolvase from the γδ transposon in <u>Vibrio cholera</u> as a permanent, heritable marker of gene expression from a chromosomal gene. The resolution system is 30 not used for excision of marker genes. Chang, L.-K. et al. ((1994, Construction of Tn917as1, a transposon useful for mutagenesis and cloning of <u>Bacillus subtilis</u> genes, Gene, 150, 129-134) describe the plasmids (pE194) containing erm-res-tnpA (transposase)-tnpR (resolvase) samt IR-res-ori colE1-AB^{R}1-AB^{R}2- 35 IR (pD917; Tn917ac1). The two res sites are there to allow the

transposon to function properly, not for excision of inter-
vening DNA.

The broad host range, gram-positive plasmid pAMβ1 (Clewell, D.
B., Yagi, Y., Dunny, G. M., Schultz, S. K. (1974) Charac-
terization of three plasmid deoxyribonucleic acid molecules in
a strain of Streptococcus faecalis: identification of a plasmid
determining erythromycin resistance. J. Bacteriol. 117, 283-
289) has been described to contain a resolution system, that
resolves plasmid multimers into monomers via a site specific
recombination event, requiring a specific plasmid encoded en-
zyme (resolvase) and a site, res, on the plasmid (Swinfield,
T.-J., Janniere, L., Ehrlich, S. D., Minton, N. P. (1991).
Characterization of a region of the Enterococcus faecalis plas-
mid pAMβ1 which enhances the segregational stability of pAMβ1-
derived cloning vectors in Bacillus subtilis. Plasmid 26, 209-
221; Janniere, L., Gruss, A., Ehrlich, S. D. (1993) Plasmids,
pp. 625-644 in Sonenshein, A. L., Hoch, J. A., Losick, R.
(eds.) Bacillus subtilis and other gram-positive bacteria:
Biochemistry, Physiology and molecular genetics. American soci-
ety for microbiology, Washington D.C.).

It has been suggested to use a site-specific recombination
system to remove a single selectable marker gene from the
genome of a bacterial cell. For instance, Dale, et al. ((1991)
Gene transfer with subsequent removal of the selection gene
from the host genome, Proc. Natl. Acad. Sci. USA, 88, 10558-
10562) describe the use of the cre/lox system for removal of
markers from transgenic plants and mentions that the use of
this system would obviate the need for different selectable
markers in subsequent rounds of gene tranfer into the same
host. Kristensen, C.S. et al. (1995), J. Bacteriol., 177, 52-
58, describe the use of the multimer resolution system of the
plasmid RP4 for the precise excision of chromosomal segments
(such as marker genes introduced with heterologous DNA) from
gram-negative bacteria. It is stated that the system is
envisaged to be of interest in the generation of chromosomal

insertions of heterologous DNA segments eventually devoid of
any selection marker.

WO 95/02058 describes a new transposon (tn5401) from B.
thuringiensis containing transposase, resolvase, and res site.
5 The transposon is used in a plasmid which contains B. thuringi-
ensis DNA (e.g. origin and toxin gene) and, flanked by res
sites, non-B. thuringiensis DNA (e.g. E. coli origin, selec-
table marker genes). The plasmid is introduced into B. thurin-
giensis. Subsequently, a plasmid expressing the resolvase is
10 introduced (e.g. a thermosensitive plasmid containing the
entrire tranposon - but only used as resolvase donor) whereby
the non-B. thuringiensis DNA is excised from the first plasmid.

Conclusions with respect to the state of the art
On the basis of the above citations, the following conclusions
15 may be made as to the state of the art:

The insertion of multiple genes of interest by transposition
was known, e.g. as described in EP 332 488. However, all
strains carrying multiple transposed sequences of interest
contain selectable markers.

20 The removal of markers via site-specific recombination was know
from either chromosome or plasmids (cf. Kristensen et al.
(1995), Eberl et al. (1994), WO 95/02058). It was known to
remove a marker introduced by transposon.

Multicopy strains without presence of heterologous, selectable
25 marker genes were known (DE 4231 764). These strains were
constructed by a cumbersome method depending on the use of the
Thy marker.

It is an object of the present invention to construct bacterial
cells which harbour a stable, fixed and well-defined copy
30 number of one or more genes of interest, without the presence
of selectable marker genes in the final strain.

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BRIEF DESCRIPTION OF THE INVENTION

The present invention is based on the surprising finding that transposition of a DNA construct which, in addition to transposase target sequences and the transposase gene necessary to achieve transposition, contains target sequences for a site-specific recombination enzyme, may be used for construction of multicopy gram-positive bacterial strains.

In a first aspect the invention relates to a DNA construct useful for integration into the genome of a host cell, the construct comprising the structure IR(1)-P-R-M2-R-IR(2) or IR(1)-R-M2-R-P-IR(2), wherein

> IR(1) and IR(2) denote transposase target sequences,
> P a DNA fragment comprising a DNA sequence of interest,
> R a target sequence for a site-specific recombination enzyme, and
> M2 a selectable marker gene,

the structure being associated with a gene (T) encoding a transposase protein capable of recognizing and interacting with the transposase target sequences IR(1) and IR(2) and located outside the structure defined by the IR(1) and IR(2) sequences.

In the present context the term "associated with" is intended to indicate that T is present on the DNA construct of the invention, but need not be in direct contact with the structure defined by IR(1) and IR(2), i.e. located within and comprising IR(1) and IR(2). Thus, linker or other sequences may be present in between the structure and T. The exact location of T is not critical as long as the gene is located outside the structure defined by IR(1) and IR(2). Thus, T may be located on either side of the structure defined by IR(1) and IR(2).

In the present context the term "selectable marker gene" is intended to indicate a DNA sequence which encodes a gene

product which provides to the host cell expressing the product
a selectable characteristic, such as resistance towards an
antibiotic. The DNA sequence M2 may further comprise one or
more regulatory elements required for or involved in the ex-
5 pression of the DNA sequence encoding the selectable marker,
such as a promoter, a terminator and the like. The regulatory
elements may be either heterologous or homologous to the DNA
sequence.

The term "transposase gene" is intended to indicate a DNA
10 sequence encoding a transposase protein, i.e. a protein which
is essential for transposition to take place. The gene may
further comprise one or more regulatory elements required for
or involved in the expression of the DNA sequence encoding the
transposase protein, such as a promoter, a terminator and the
15 like. The regulatory element(s) may be either heterologous or
homologous to the DNA sequence.

The term "transposase target sequence" is intended to indicate
DNA sequences recognized by the transposase protein encoded by
the transposase gene T. The transposase target sequences IR(1)
20 and IR(2) shall contain sufficient DNA derived from transposon
terminal sequences to retain the function of these sequences in
allowing transposition of the structure comprising and located
within these sequences upon expression of the transposase
encoded by T. The minimal sequences that are sufficient for
25 transposition vary in composition and length depending on which
transposon the system is developed from. For instance, for Tn10
derived transposase target sequences, either 23 or 42 basepairs
are enough to allow transposition (Kleckner, N. (1988) Trans-
poson Tn10. pp 227-268 in Berg, D. E., and Howe, M. M. (eds).
30 Mobile DNA. American society for microbiology, Washington,
D.C.).

In the present context the "target sequence for a site-specific
recombination enzyme" is intended to indicate a DNA sequence
which is recognized by a site-specific recombinase enzyme

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(discussed in detail further below). The target sequences may be, but need not be identical. Thus, variations between the sequences may occur as long as the recombinase is capable of recognizing and interacting with the sequences.

5 The term "DNA sequence of interest" is used to indicate a sequence which codes for a desired RNA or protein product (heterologous or native to the host cell) or which in itself provides the host cell with a desired property, e.g. a mutant phenotype. When appropriate, the DNA sequence may also comprise 10 one or more regulatory elements required for or involved in the expression of the DNA sequence encoding the desired RNA or protein product, such as a promoter, a terminator and the like. The regulatory element(s) may be either heterologous or homologous to the DNA sequence.

15 The novel DNA construct of the invention has been found of particular use in construction of strains which in their genome comprise more than one, and preferably multiple, randomly located copies of integrated DNA sequences, which cells in preferred aspects are free from genes encoding a selectable 20 marker the presence of which is undesirable, e.g., from an environmental point of view. It will be noted that the DNA construct of the invention is different from those provided in EP 485 701 in that the selectable marker gene in the present invention is located outside the structure flanked by trans- 25 posase target sequences. This location is essential for constructing marker-free cells in accordance with the present invention.

An alternative approach to using the above described recombinase system for deleting a marker gene used in the construction 30 of modified cells by transposition is to effect the deletion of the marker gene by homologous recombination. Accordingly, in a second aspect of the present invention relates to a DNA construct is provided which comprises the structure IR(1)-P-R'-M2-R''-IR(2) or IR(1)-R'-M2-R''-P-IR(2), wherein

IR(1) and IR(2) denote transposase target sequences,
P a DNA fragment comprising a DNA sequence of inter-
est,
R' and R'', respectively, a DNA sequence which is pro-
vided in a parallel repeat on either side of the
selectable marker gene M2, and
M2 a selectable marker gene,

the structure being associated with a gene (T) encoding a
transposase protein capable of recognizing and interacting with
the transposase target sequences IR(1) and IR(2) and located
outside the structure defined by the IR(1) and IR(2) sequences,
i.e. outside the structure IR(1)-P-R'-M2-R''-IR(2) or IR(1)-R'-
M2-R''-P-IR(2).

The term "provided in a parallel repeat" is intended to
indicate that the DNA sequences R' and R'' flanking M2 are
sufficiently homologous to allow for homologous recombination
to take place between the sequences. Preferably, each of the
sequences R' and R'' comprises a substantially identical segment
of at least 20 nucleotides, more preferably of at least 50
nucleotides, such as 50-100 nucleotides, and even more pre-
ferably at least 500 (such as 500-1000 nucleotides) and most
preferably at least 1000 nucleotides, such as 1000-3000
nucleotides.

The presence of the target sequences for a site-specific
recombination enzyme (first aspect) or of the parallel repeats
(second aspect) enables the specific deletion of the marker
gene M2 from the genome of a host cell which in its genome have
received the marker gene by transposition. More specifically,
the DNA constructs according to the first and second aspect of
the invention, respecitively, in which the marker gene M2 is
located inbetween target sequences for a site-specific recombi-
nation enzyme or inbetween parallel repeat sequences, is
particularly useful for the construction of marker-free,
multicopy strains (i.e. bacterial cells which in their genome

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comprise multiple copies of an integrated DNA sequence of interest). In short each of the DNA constructs is intended to be used in a two-step integration process, wherein, in the first step, genomic integration of the fragment IR(1)-P-R-M2-R-IR(2) or IR(1)-R-M2-R-P-IR(2) (the first aspect), or IR(1)-P-R'-M2-R''-IR(2) or IR(1)-R'-M2-R''-P-IR(2) (the second aspect), is accomplished by transposition and selection for $M2^+$-cells, and, in the second step, the selectable marker gene M2 is eliminated by a site-specific recombinase enzyme provided in trans and interacting with the target sequences R flanking the marker gene M2 (the first aspect) or by recombination between the parallel repeat sequences R' and R'' (the second aspect).

In the present context the term "marker-free" is intended to indicate that the cell having integrated multiple copies of a DNA sequence of interest does not express a selectable marker, such as an antibiotic resistance, the presence of which is undesired in the cell, which marker is necessary for or advantageously used in the construction of the cell.

DETAILED DESCRIPTION OF THE INVENTION

In the following the various aspects of the invention is discussed in further detail.

The DNA construct of the invention

In addition to the DNA constructs according to the first and second aspect of the invention, which are described above, in a third aspect, the present invention relates to a DNA construct comprising the structure IR(1)-R-M2-T-R-P-IR(2), IR(1)-P-R-M2-T-R-IR(2), IR(1)-R-T-M2-R-P-IR(2) or IR(1)-P-R-T-M2-R-IR(2), wherein

IR(1) and IR(2) denote transposase target sequences,
P a DNA sequence of interest,
R a target sequence for a site-specific recombination enzyme,

M2 a selectable marker gene, and

T a transposase gene T.

As compared to the DNA construct according to the first aspect
of the invention the DNA construct of the third aspect compri-
ses the transposase gene T inside the structure defined by
IR(1) and IR(2) rather than outside said structure.

Analogously, in a fourth aspect the invention relates to a DNA
construct comprising the structure IR(1)-R'-M2-T-R''-P-IR(2),
IR(1)-P-R'-M2-T-R''-IR(2), IR(1)-R'-T-M2-R''-P-IR(2) or IR(1)-
P-R'-T-M2-R''-IR(2), wherein

IR(1) and IR(2) denote transposase target sequences,

P a DNA sequence of interest,

R' and R'' parallel repeat sequences,

M2 a selectable marker gene, and

T a transposase gene T.

The DNA construct of the invention is preferably one, which is
capable of being transposed in cells of gram-positive bacteria,
in particular strains of <u>Bacillus</u>.

Selectable marker

In order to facilitate selection for cells in which the part of
the DNA construct of the invention located within and compri-
sing the inverted repeat sequences IR(1) and IR(2) (e.g. the
structure IR(1)-P-R-M2-R-IR(2)   or   IR(1)-R-M2-R-P-IR(2)   in
connection with the first aspect of the invention) has been
integrated in the genome, it is advantageous that the DNA
construct comprises a further selectable marker gene M1 outside
the structure defined by and comprising IR(1) and IR(2). In
this case, the DNA construct comprises a first selectable
marker gene M1 present outside the structure defined by and
comprising IR(1) and IR(2) and a second marker M2 within said
structure. Thereby, when M1 is different from M2, it is

possible to make a two step selection when the DNA construct of
the invention is transposed into the genome of a recipient
cell, the first step selecting for cells in which the DNA con-
struct has been introduced (M1⁺, M2⁺ cells) and a second step
selecting for cells in which the structure defined by IR(1) and
IR(2) comprising the marker gene M2 has been integrated into
the genome and the remaining part of the DNA construct (car-
rying M1) has been lost (M1⁻, M2⁺). Subsequently, the marker
gene M2 may be eliminated from the resulting cells by the
action of a resolvase protein.

The selectable marker may be a gene coding for a product which
confers antibiotic resistance to a cell expressing the gene or
a non-antibiotic marker gene, such as a gene releiving other
types of growth inhibition, i.e. a marker gene which allow
cells containing the gene to grow under otherwise growth-
inhibitory conditions. Examples of such genes include a gene
which confers prototrophy to an auxotrophic strain, e.g. <u>dal</u>
genes introduced in a <u>dal</u>⁻ strain (cf. B. Diderichsen in
<u>Bacillus: Molecular Genetics and Biotechnology Applications</u>,
A.T. Ganesan and J.A. Hoch, Eds., Academic Press, 1986, pp. 35-
46) or a <u>thy</u> gene introduced in a <u>thy</u>⁻-cell (cf. Gryczan and
Dubnau (1982), Gene, 20, 459-469) or a gene which enables a
cell harbouring the gene to grow under specific conditions such
as an <u>amdS</u> gene, the expression of which enables a cell
harbouring the gene to grow on acetamide as the only nitrogen
or carbon source (e.g. as described in EP 635 574), or a gene
which confers resistance towards a heavy metal (e.g. arsenite,
arsenate, antimony, cadmium or organo-mercurial compounds) to
a cell expressing the gene. Cells surviving under these
conditions will either be cells containing the introduced DNA
construct in an extrachromosomal state or cells in which the
above structure has been integrated. Alternatively, the
selectable marker gene may be one conferring immunity to a cell
expressing the gene.

15

The DNA sequence of interest

The DNA sequence of interest present in the DNA construct of
the invention may be a DNA sequence having or encoding any
function. For instance, the DNA sequence may comprise a
5 sequence encoding a structural or regulatory protein, or may
comprise a regulatory sequence such as a promoter. Alternati-
vely, the inserted sequence may be one not known to possess any
biological function, which can be used to interrupt a cellular
function, e.g. by inserting itself within an essential gene
10 thereby interrupting the function of the gene. The DNA sequence
of interest may be a gene and thus associated with the neces-
sary regulatory elements required for its expression including
a promoter, a terminator or a ribosome binding site.

As will be understood from the above the present invention is
15 of particular use in constructing bacterial cells comprising
multiple copies of a DNA sequence of interest. Such multicopy
strains are of particular interest for the industrial produc-
tion of polypeptides of interest and, accordingly, in a highly
preferred embodiment the DNA sequence of interest encodes a
20 polypeptide of interest.

The polypeptide may be a translocated polypeptide, i.e. a
polypeptide which, when expressed, carries a signal sequence
which enables it to be translocated across the cell membrane.
In particular, the translocated polypeptide may be a secreted
25 polypeptide or a polypeptide involved in the secretory machi-
nery of the bacterial cell in question.

The polypeptide, whether secreted or not, may be an enzyme,
e.g., selected from an amylolytic enzyme, a lipolytic enzyme,
a proteolytic enzyme, a cellulytic enzyme, an oxidoreductase or
30 a plant cell-wall degrading enzyme. Examples of such enzymes
include AMG, amylase, lipase, cutinase, esterase, cellulase,
hemicellulase, protease, peroxidase, laccase, phenoloxidase,
catalase, glucose oxidase, phytase, lyase, pectinase, glucosi-

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dase, mannosidase, isomerase, invertase, trasferase, ribonucle-
ase, galactosidase and chitinase. Alternatively, the secreted
polypeptide may be a hormone, a growth factor, a receptor or
the like.

5 A preferred example of a translocated polypeptide of the
secretory pathway is PrsA (WO 94/19471) which, when overex-
pressed in cells of Bacillus, has been found to result in an
increased secretion of a secreted polypeptide of interest from
such cells.

10 ## Transposase and transposase recognition sequences

It will be understood that the transposase gene and the
transposase target sequences must be chosen so as to be capable
of functioning together. In the present invention any trans-
posase recognition sequence may be used together with the
15 corresponding transposase. Typically, the transposase gene and
the transposase target sequences are derived from the same
class of transposons (cf the Background of the Invention
section above), and preferably from the same transposon. The
transposase recognition sequences may be inverted repeat
20 sequences, e.g. derived from Tn1, Tn2, Tn3, Tn5, Tn9, Tn10 and
Tn903.

Alternatively, the transposase recognition sequences are free
from inverted repeats. Examples of such sequences are those
derived from the transposase recognition sequences of bac-
25 teriophage Mu and related transposons. When the transposase
recognition sequences are derived from bacteriophage Mu it may
be necessary to supply an accessory protein in addition to the
transposase (cf the Background of the Invention section above).

The transposase gene and the transposase target sequences may
30 be derived from naturally occurring transposons either by being
isolated from the relevant source by use of standard techniques
or by being synthesized on the basis of known sequences. It

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will be understood that functional analogues or derivatives of
naturally occurring sequences may be used as long as they are
capable of mediating the transposition. The functional analogu-
es or derivatives may be prepared synthetically and may differ
5 from the wild type sequence in one or more nucleotides.

Resolvase and resolvase target sequences

It will be understood that the essential feature according to
the second aspect of the invention is the use of a site-
specific recombination system to specifically delete the marker
10 gene M2, once transposition into the host genome has taken
place. Several site-specific recombination systems are known
(cf the Background of the Invention section above) all of which
are contemplated to be useful for use in the present invention.
For the purpose of the present invention, the preferred use is
15 of a system which consists of only two elements: a site
specific recombinase enzyme and a target sequence for said
enzyme.

Examples of such systems are the pAMβ1 resolvase having as
target sequence the pAMβ1 res sequence (Janniere, L., Gruss,
20 A., Ehrlich, S. D. (1993), Plasmids, pp. 625-644 in Sonenshein,
A. L., Hoch, J. A., Losick, R. (eds.) Bacillus subtilis and
other gram-positive bacteria: Biochemistry, Physiology and
molecular genetics, American society for microbiology, Washing-
ton D.C.) and the phage P1 Cre enzyme having as target sequence
25 the P1 lox site (Hasan, N., Koob, M., Szybalski, W. (1994).
Escherichia coli genome targeting, I. Cre-lox-mediated in vitro
generation of ori⁻ plasmids and their in vivo chromosomal inte-
gration and retrieval, Gene, 150, 51-56).

It will be understood that the DNA sequence encoding the
30 recombinase enzyme and the target sequence for this enzyme may
be derived from naturally occurring systems (as described
above) either by being isolated from the relevant source by use
of standard techniques or by being synthesized on the basis of

known sequences. It will be understood that functional analogu-
es or derivatives of naturally occurring sequences may be used
as long as they are capable of functioning in the intended
manner. The functional analogues or derivatives may be prepared
5 synthetically and may differ from the wild type sequence in one
or more nucleotides.

For some purposes it may be advantageous to introduce the gene
encoding the recombinase enzyme (when provided in trans) into
the cell by use of a naturally occurring plasmid, such as
10 pAMβ1, comprising the gene.

The DNA sequence encoding the recombinase enzyme is preferably
provided in trans. However, it may be advantagous that the DNA
sequence is present in the vector which carries the DNA
construct of the invention. In this case the DNA sequence is
15 preferably located in the part of the vector which is integra-
ted into the genome of the host cell, and most preferably on
the part of the DNA which after integration is excised from the
genome due to the action of the recombinase enzyme. In addi-
tion, the DNA sequence should be located under the control of
20 an inducible or regutable promoter such as a temperature
inducible promoter or a xylose inducible promoter or the SPAC
promoter, so that the expression from the DNA sequence could be
controlled to take place only after transposition has taken
place. If the DNA sequence encoding the recombinase enzyme is
25 provided in cis and under the control of a regutable promoter
the method of the invention may be accomplished using only one
transformation step.

Furthermore, the gene encoding the site specific recombination
enzyme may be engineered by methods well known in the art to
30 allow its proper expression in the host strain. It may by
transferred onto a temperature-sensitive or otherwise conditio-
nal replicon, to allow for easy loss of the gene from the host
cell once the site-specific recombination event has occurred.

Homologous recombination in combination with a counterselectable marker

Although the selectable marker gene may be deleted by homologous recombination between the DNA sequences R' and R'' when
5 using the DNA construct according to the second aspect of the invention such homologous recombination may occur with a very low frequency for which screening may be difficult to achieve in actual practice. In order to avoid this practical problem, it may be advantageous that the selectable marker gene M2 is a
10 gene which in the host cell of choice can be counterselected. In other words, it may be desirable to use a marker, the presence as well as the absence of which can be selected. For instance, the marker gene may be one conferring prototrophy to an auxotrophic cell, e.g. a thy gene for use in a thy⁻-cell
15 (Gryczan, T.J., and Dubnau, D. (1982), Direct selection of recombinant plasmids in Bacillus subtilis, Gene, 20, 459-469), or a gene which enables a cell expressing the gene to grow on a specific substrate, e.g. amdS, the expression of which allows cells to grow on acetamide as the only nitrogen or carbon
20 source (EP 635 574). When the thy gene is used a selectable marker M2 the DNA construct according to the second aspect is introduced into a thy⁻-cell allowing for selection of cells having received the construct (first step) and in a second step the resulting cells are subjected to growth on a medium which
25 cannot tolerate the presence of the thy gene, thereby allowing for selection of cells having lost the thy gene by homologous recombination (the principle of using a thy gene as a counterselectable marker is described in detail by Gryczan and Dubnau op. cit.). Analogously, the amdS gene may be used in a two step
30 procedure, wherein in a first step selection is made for amdS-containing cells by growth on acetamide as the only carbon or nitrogen source, and in a second step counterselection is made by growing the cells on a medium containing flouracetamide and urea as nitrogen sources (cf EP 635 574) whereby cells are
35 selected which do not contain the amdS gene.

Construction of the DNA construct of the invention

A DNA construct of the invention may conveniently be con-
structed on the basis of naturally occurring or otherwise
existing transposon delivery vectors. The transposon delivery
5 vectors described in the literature contains a transposase
gene, if needed modified so as to be expressed in the intended
host organism, and a transposable cassette, essentially
containing a resistance gene flanked by sufficient DNA derived
from the ends (e.g. inverted repeat sequences) of the original
10 transposon to enable the transposition of the resistance gene
into the host genome.

In one embodiment the DNA construct of the invention may be
prepared by modification of such transposon delivery vectors so
as to contain, within the structure that by transposition
15 integrates into the host genome, a DNA sequence of interest,
either replacing or in addition to the antibiotic resistance
gene. It is further possible to replace the antibiotic re-
sistance gene with any other selectable marker gene. The
modification of the transposon delivery vectors may be ac-
20 complished by methods known in the art. In an alternative em-
bodiment the DNA construct of the invention is prepared by
joining each of the isolated elements to be comprised by the
DNA construct using methods known in the art.

When the selectable marker gene is to be eliminated from the
25 cells in which the DNA construct has been integrated the marker
should be flanked by resolvase target sequences or by parallel-
ly repeated homologous DNA sequences. These sequences may be
inserted into the DNA construct prepared as described above by
methods known in the art. It is important that the gene of
30 interest is located outside the structure flanked by these
target sequences.

The vector of the invention

Origin of replication

For use in the present invention it is generally preferred that the DNA construct of the invention is linked to an origin of
5 replication, i.e. present on a vector. In the present context the term "vector" is intended to denote a DNA molecule capable of functioning as an autonomously replicating extrachromosomal element. The vector may, e.g., be a plasmid or a bacteriophage.

The origin of replication should not be located between the
10 transposase recognition sequences IR(1) and IR(2) since such location would result in the origin of replication being integrated into the genome of the host cell. Genomic integration of an origin of replication is undesirable from a stability point of view.

15 Except for the above the location of the origin of replication is not critical. Thus, in a DNA construct of the invention the origin of replication may be located on either side of "T" and "M1"(when present).

If it is desired to improve the efficiency with which cells
20 harbouring an integrated DNA construct can be isolated, the DNA construct may be present on a vector comprising a conditional origin of replication. In other words a vector may be used which is able to replicate under certain (permissive) conditions and unable to replicate under other (non-permissive)
25 conditions. The vector may, for instance, be one which is temperature-sensitive for replication. Thus, in an embodiment of the method of the invention, the vector comprising the DNA construct to be integrated is one which is unable to replicate at increased temperatures, which yet permit growth of the host
30 cell. The bacterial cells are initially cultured at a temperature permitting vector replication and subsequently, after integration of the DNA construct of the invention, into the

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bacterial genome may have taken place, cultured at a tempera-
ture which does not permit vector replication so that the
vector introduced into the cell is lost from the cells. The
cultivation at the non-permissive temperature is conducted
5 under selective conditions to ensure that only cells containing
the integrated DNA construct will survive.

Another way of increasing the efficiency of integration and
subsequent loss of the vector from the cells may be to treat
the cells transformed with the vector with a plasmid-curing
10 agent, e.g. novobiocin (Gadó, I. et al., 1987. Zbl. Bakt. Hyg.
A. 265, 136-145), after culturing the host cells under selec-
tive conditions as described above.

A still further type of a conditional replication origin may be
one of a limited host-range plasmid, i.e. a plasmid which can
15 only replicate in a limited number of microbial species. For
the present purpose an origin of replication should be chosen
which is derived from a limited host-range plasmid which is
unable to replicate in the recipient cell into which the DNA
cnstruct is to be transposed.

20 When the DNA construct of the invention is constructed by
modification of transposon delivery vectors the origin of
replication normally present on these vectors may be used.
Otherwise, the origin of replication may be provided, con-
veniently by inserting the DNA construct into a vector suitable
25 for the introduction into the intended host cell. The integra-
tion of the transposable cassette into the genome of the host
cell may be accomplished following the procedures described in
the literature (Petit et al., 1990 op. cit.) for use of
transposon delivery vectors for mutagenesis.

30 Cis-acting conjugational elements

It may be desirable to introduce the vector of the invention
carrying a DNA construct of the invention into the recipient

cell in question by means of conjugation (further described in
the section below entitled "Construction of single or multi-
copy, optionally marker-free strains"). For this purpose the
DNA construct or vector comprises a so-called cis-acting DNA
5 sequence which is required for conjugation to take place.

Accordingly, when a vector carrying a DNA construct of the
invention is to be introduced into a recipient cell by con-
jugation the DNA construct or vector further comprises a cis-
acting DNA sequence required for transfer of a plasmid compri-
10 sing the DNA construct.

The cis-acting DNA sequence may conveniently be any DNA
sequence or DNA site capable of mediating mobilization, a
preferred example being oriT of the tetracycline resistance
plasmid pBC16 or of the Staphylococcus aureus kanamycin re-
15 sistance plasmid pUB110 (as described by Sellinger et al.,
Journal of Bacteriology, June 1990, pp. 3290-3297) or a func-
tional analogue or part of oriT.

For all types of vectors carrying a DNA construct of the in-
vention (harbouring any of the structures as defined in
20 connection with the first, second, third and fourth aspect of
the invention) the cis-acting sequence may be located on the
vector, but outside the structure defined by and comprising
IR(1) and IR(2),whereby the sequence will be excised together
with the transposon delivery vector. Thus, the cis-acting DNA
25 sequence may be located on either side of said structure and of
the DNA sequence T and the DNA sequence M1, when present.

In an preferred embodiment of the first, second, third and
fourth aspect of the invention, the cis-acting sequence is
located in the part of the structure located within and compri-
30 sing the resolvase target sequences R and the parallel repeat
sequences R' and R'', respectively. Thereby, the cis-acting
sequence may be excised - together with the selectable marker
gene M2 and optionally T (second and fourth aspect of the

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invention) - from the genome of the cell in which the DNA structure has been integrated.

Construction of single or multicopy, optionally marker-free strains

As it will be understood the construction of multicopy, marker-free production strains may be achieved by use of the above described DNA constructs allowing the stepwise integration of one or more copies of a DNA sequence of interest into the genome of a bacterial cell, which method comprises repeated steps of transposition followed by site-specific marker deletion.

Accordingly, in further aspects the invention relates to methods of constructing a bacterial cell of the invention by use of the DNA construct according to the first, second, third and fourth aspect, respectively, as further defined above.

Accordingly, in a further aspect the invention relates to a method of constructing a bacterial cell, which in its genomic DNA has integrated more than one copy of a DNA sequence of interest, and which is free from a DNA sequence encoding an undesired selectable marker, which method comprises

a) introducing a first vector comprising a DNA construct according to the first aspect of the invention comprising the structure IR(1)-P-R-M2-R-IR(2) or IR(1)-R-M2-R-P-IR(2) in association with a tranposase gene T and optionally a selectable marker gene M1 into the host cell, in which R denotes a target sequence for a site-specific recombination enzyme,

b) selecting for cells being $M2^+$ and optionally M1-, which in their genome comprises the structure IR(1)-P-R-M2-R-IR(2) or IR(1)-R-M2-R-P-IR(2),

25

c) introducing a second vector comprising a DNA sequence encoding a site specific recombinase into the cells selected in step b) so as to excise the structure R-M2 or M2-R from the genome of the cell whereby cells are obtained having integrated
5 the structure IR(1)-R-P-IR(2) or IR(1)-P-R-IR(2),

d) curing the cells resulting from step c) for the second plasmid, and optionally

e) repeating steps a-d) one or more times to produce bacterial cells comprising one or more additional copies of the structure
10 IR(1)-R-P-IR(2) or IR(1)-P-R-IR(2).

In a still further aspect the invention relates to a method of constructing a bacterial cell, which in its genomic DNA has integrated more than one copy of a DNA sequence of interest, and which is free from a DNA sequence encoding an undesired
15 selectable marker, which method comprises

a) introducing a first vector comprising a DNA construct according to the third aspect of the invention comprising the structure   IR(1)-R-M2-T-R-P-IR(2),   IR(1)-P-R-M2-T-R-IR(2), IR(1)-R-T-M2-R-P-IR(2) or IR(1)-P-R-T-M2-R-IR(2) optionally in
20 association with a selectable marker gene M1 into the host cell,

b) selecting for cells being M1⁻ (if M1 was present on the vector) and M2⁺, which cells in their genome comprises one of the structures identified in a),

25 c) selecting for cells having an increased number of copies of the marker gene M2,

d) introducing a second vector comprising a DNA sequence encoding a site specific recombinase into the cells selected in step b) so as to excise the structure R-M2-T, R-T-M2, M2-T-R or
30 T-R-M2 from the genome of the cell whereby cells are obtained

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having integrated the structure IR(1)-R-P-IR(2) or IR(1)-P-R-IR(2),

e) curing the cells resulting from step d) for the second plasmid, and optionally

5 f) repeating steps a-e one or more times to produce bacterial cells comprising one or more additional copies of the structure IR(1)-R-P-IR(2) or IR(1)-P-R-IR(2).

In a still further aspect the invetion relates to a method for constructing a bacterial cell, which in its genomic DNA has 10 integrated more than one copy of a DNA sequence of interest, and which is free from a DNA sequence encoding an undesired selectable marker, which method comprises

a) introducing a first vector comprising a DNA construct according to the second aspect of the invention comprising the 15 structure IR(1)-P-R'-M2-R''-IR(2) or IR(1)-R'-M2-R''-P-IR(2) in association with a tranposase gene T and optionally a selec- table marker gene M1 into the host cell, in which R' and R'' denote parallel repeat sequences,

b) selecting for cells being M1⁻ (if M1 was present on the 20 vector) and M2⁺, which cells in their genome comprises the structure IR(1)-P-R'-M2-R''-IR(2) or IR(1)-R'-M2-R''-P-IR(2),

c) allowing homologous recombination between the DNA sequences R' and R'' to take place so as to excise the selectable marker gene M2, whereby cells are obtained having integrated the 25 structure IR(1)-R'/R''-P-IR(2) or IR(1)-P-R'/R''-IR(2) (wherein R'/R'' denotes the common recombination sequence), and op- tionally

d) repeating steps a-c one or more times to produce bacterial cells comprising one or more additional copies of the DNA 30 structure IR(1)-R'/R''-P-IR(2) or IR(1)-P-R'/R''-IR(2).

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In a still further aspect the invention relates to a method for constructing a bacterial cell, which in its genomic DNA has integrated more than one copy of a DNA sequence of interest, and which is free from a DNA sequence encoding an undesired
5 selectable marker, which method comprises

a) introducing a first vector comprising a DNA construct according to the fourth aspect of the invention comprising the structure IR(1)-R'-M2-T-R''-P-IR(2), IR(1)-P-R'-M2-T-R''-IR(2), IR(1)-R'-T-M2-R''-P-IR(2) or IR(1)-P-R'-T-M2-R''-IR(2) op-
10 tionally in association with a selectable marker gene M1 into the host cell, in which R' and R'' denote parallel repeat sequences,

b) selecting for cells being M1⁻ (if M1 was present on the vector) and M2⁺, which in their genome comprises the relevant
15 structure identified in a),

c) selecting for cells having an increased number of copies of the selectable marker gene M2,

d) allowing homologous recombination between the DNA sequences R' and R'' to take place so as to excise the selectable marker
20 gene M2 and the transposase gene T, whereby cells are obtained having integrated the structure IR(1)-R'/R''-P-IR(2) or IR(1)-P-R'/R''-IR(2) (wherein R'/R'' denotes the common recombination sequence), and optionally

e) repeating steps a-d one or more times to produce bacterial
25 cells comprising one or more additional copies of the DNA structure IR(1)-R'/R''-P-IR(2) or IR(1)-P-R'/R''-IR(2).

It will be noted that integration of the DNA construct according to the first and third aspect, respectively, results in the same host cell harbouring the structure IR(1)-R-P-IR(2) or
30 IR(1)-P-R-IR(2) and that integration of the DNA construct according to the second and fourth aspect, respectively,

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results in the same host cell harbouring the structure IR(1)-
R'/R''-P-IR(2) or IR(1)-P-R'/R''-IR(2).

In **step a)** of any of the above methods, the vector may be
introduced into the bacterial cell by any method suitable for
5 the bacterial cell in question. A variety of methods for
introduction of DNA into bacterial cells are well known in the
art. These include the use of competent cells (by chemical
treatment, or "natural" competence), protoplast transformation,
conjugation, electroporation, transduction, or ballistic
10 transformation. While conjugation may be accomplished by use of
well-known self-transmissible plasmids as cloning vectors (e.g.
the plasmid pLS20), the following specific conjugation method
has been found to be generally applicable for introduction of
DNA into cells, e.g. of *Bacillus* sp., which cannot or only with
15 difficulty receive DNA by conventional methods such as pro-
toplast fusion and the like.

More specifically, conjugation is conveniently achieved by a
method, in which a population of bacterial donor cells har-
bouring i) a plasmid comprising a DNA construct of the in-
20 vention and at least one cis-acting DNA sequence required for
the transfer of said plasmid by conjugation in the presence of
a trans-acting mobilizing element, and ii) at least one DNA
sequence encoding said trans-acting mobilizing element, and a
population of recipient cells are mixed under conditions
25 allowing the plasmid to be transferred from the population of
the donor cells to the population of recipient cells by con-
jugation. The population of donor cells may, e.g., be a
population of cells of *E. coli* or a *Bacillus* sp., examples of
which are mentioned further below. The recipient cells are
30 preferably cells of a *Bacillus* sp. such as the ones mentioned
further below. Of particular interest is recipient cells of *B.
licheniformis*, *B. amyloliquefaciens* and *B. lentus* and non-
competent cells of *B. subtilis*. The cis-acting DNA sequence and
preferred locations thereof in the DNA construct or vector of

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the invention are described in the section above entitled "Cis-
acting conjugational elements".

The term "trans-acting mobilizing element" in intended to
indicate a protein mediating conjugative transfer of DNA
sequences containing the cis-acting DNA sequence defined above.
The trans-acting mobilizing element may be a protein encoded by
a conjugational plasmid, such as the Bacillus sp. plasmid pLS20
(Koehler and Thorne, Journal of Bacteriology, Nov. 1987, pp.
5771-5278), or a part or derivative thereof, or may be a
protein encoded by a DNA sequence such as orf-β of the plasmids
pBC16 or pUB110 (Sellinger et al., Journal of Bacteriology,
June 1990, pp. 3290-3297) or a functional analogue or part
thereof. It will be understood that since the mobilizing
element is acting in trans it may be encoded by DNA present in
the genome of the donor cell or on a second plasmid present in
said donor cell.

The mixing of the cells is conveniently performed by mixing the
donor and recipient cells and allow them to stand for at least
4 hours at 30-37°C and selecting for recipient cells having
received the DNA construct. See Selinger et al., 1990, for
further reference.

The cultivation conditions to be used in step b) depend, inter
alia, on the kind of selectable marker. For instance, when the
selectable marker encoded by M2 is an antibiotic resistance the
cultivation of b) is performed in the presence of a suitable
dosage of the antibiotic in question so as to select cells
having received and expressing M2.

Selection and screening are considerably facilitated by the
presence of the antibiotic resistance markers M1 and M2 on the
DNA construct which is introduced into the recepient host cell.
The marker M1 being present on the "vector part" of the DNA
construct, i.e. the part of the construct outside the structure
defined by IR(1) and IR(2), whereas the marker gene M2 is

30

present within said structure. However, it may be noted that
succesfull transposition can be obtained also when the DNA
construct comprises only one selectable marker, i.e. either M1
or M2.

5 Following a suitable growth regime, e.g. as given in the li-
terature for pHV1248 (Petit, M.-A., Bruand, C., Janniere, L.,
Ehrlich, S. D. (1990), Tn10-derived transposons active in Ba-
cillus subtilis, J. Bacteriol., 172, 6736-6740), strains con-
taining M2 are selected on plates containing the proper antibi-
10 otic, and screened by replica plating for the absence of M1.
Such strains no longer harbour the transposon delivery vector,
and has the transposable structure integrated into their
genome. Integration of the DNA construct by transposition may
occur at random positions throughout the genome of the host
15 cell. Thus, when multicopy cells are produced the integrated
copies will be located at random, separate positions of the
cell which contributes to increasing the genetic stability of
the cell.

The presence of P may be evaluated by methods known in the art,
20 e.g., as appropriate, by southern analysis, PCR amplification,
or phenotypic expression of P. When the DNA construct to be
introduced into the cell harbours a selectable marker gene M1
the loss of the M1 phenotype is used to indicate loss of the
vector part of the introduced DNA once transposition has taken
25 place.

When the DNA construct according to the first or third aspect
of the invention is used (i.e. a DNA construction comprising
target sequences for a site-specific recombinase enzyme) a
subsequent step is to introduce a second vector expressing the
30 site-specific recombination enzyme cognate to the target sequ-
ences flanking M2 into the strain having recieved the first
vector. The activity of the recombination enzyme recombines the
two target sequences, which in the genome flanks M2 and
optionally T, thus deleting this marker gene and optionally T,

an event which is easily detected by screening, e.g. by replica
plating.

Once M2-free strains are obtained, these are cured for the
plasmid expressing the recombination enzyme, e.g. by propaga-
5 tion at non-permissive temperature if the vector is a tempera-
ture-sensitive replicon. Alternatively, the vector is allowed
a certain time of residence in the host, which is then cured
for the vector, and the plasmid free cells screened for loss of
M2.

10 The result of this process is a strain that contains one trans-
posed copy of the gene of interest, but no marker gene.
This strain is now used as a host strain in a new round of
transposition and marker deletion, in which it is possible to
employ exactly the same modified transposon delivery vector and
15 recombination enzyme encoding vector as in the first round,
i.e.
i) the modified transposon delivery vector is introduced into
the host strain,

ii) strains containing a transposed copy of the DNA sequence of
20 interest plus the marker gene M2, and free of transposon deli-
very vector, are obtained as described above,

iii) the vector expressing the cognate site specific recombi-
nation enzyme is introduced into these strains (in case of the
DNA construct according to the third aspect), and

25 iv) strains from which the marker gene M2 and optionally T is
deleted due to the activity of the recombination enzyme (when
constructed from a DNA construct according to the first or
third aspect) or homologous recombination (when constructed
from a DNA construct according to the second or fourth aspect)
30 are isolated as above, and plasmid free versions of such
strains obtained.

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The result of this is now a strain containing two transposed copies of the gene of interest, but no marker gene.

This can again serve as host strain in a new round of transposition and marker deletion, and the process can be repeated
5 essentially an infinite number of times.

If the DNA sequence encoding the recombinase enzyme is provided in cis under a regulatable promoter (as discussed above), only one transformation step need to be done in each round. In practice when transposition has been accomplished the regula-
10 table promoter is switched on and the recombinase enzyme expressed.

Marker deletion by the site-specific recombination enzyme leaves one copy of the cognate target sequence in the chromosome. Subsequent transposition events brings in new copies of this
15 target sequence. As transposition can occur to a substantial number of sites within the genome, approaching random integration, the new target sequences will with high probability be situated at a considerable distance from the first one. It is likely that the site-specific recombination enzyme may function
20 less efficiently on very separated sequences. It is furthermore likely that the deletion of a substantial amount of genomic DNA, which would result from such a recombination event, would be lethal, so strains where this has taken place would not be isolated by the described procedure.

25 An analogous process is carried out in connection with strains produced by use of a DNA construct according to the second or fourth aspect of the invention. In this case the excision of the marker gene M2 and, when relevant T, is obtained by homologous recombination between the sequences R' and R''.

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The cell of the invention

In a further aspect the invention relates to a bacterial cell, which in its genome has integrated more than one copy of a DNA construct comprising the following structure IR(1)-P-IR(2), in
5 which

IR(1) and IR(2) denote transposase target sequences, and

P a DNA sequence of interest, wherein the structure IR(1)-P-IR(2) does not encode an undesired selectable marker. The structure further comprises a recombination target sequence R
10 or the common homologous recombination sequence R'/R'' inbetween IR(1) and IR(2).

In the present context the term "genome" is intended to indicate the constituent DNA of the cell comprising the chromosome and stably inherited extrachromosomal elements.

15 In the present context the cell into which the DNA construct is to be integrated is denoted host cell, host strain, recipient strain or cell, modified cell or the like. It will be understood that these terms are used interchangeably.

The presence of IR(1) and IR(2) in the genome of the cell is
20 not essential for the function of the cell, but is evidence to the fact that the cell has been constructed by transposition, i.e. by use of transposons. Thus, only cells having been modified by transposition comprise the above structure. If necessary, the IR(1) and/or IR(2) may be deleted or inactivated
25 from the cell by conventional means.

In a further aspect the invention relates to a bacterial cell, which in its genome has integrated at least two copies of a DNA construct comprising the following structure IR(1)-P-IR(2), in which
30 IR(1) and IR(2) denote transposase target sequences, and

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P a DNA sequence of interest, the structure further comprising a recombination target sequence R or the common homologous recombination sequence R'/R'' inbetween IR(1) and IR(2).

It is presently believed that the present disclosure is the
5 first description of the use of transposition for construction of multicopy grampositive cells, in particular <u>Bacillus</u> cells. The presence of multiple, normally randomly located copies of the structure IR(1)-P-IR(2) further comprising R or R'/R'' in the genome of a cell shows that the cell has been constructed
10 by transposition.

From the above disclosure it will be apparent that the present invention is a very convenient and efficient method of constructing multicopy gram-positive bacterial strains, i.e. bacterial cells which integrated in their genome comprises
15 multiple copies of a DNA sequence of interest. The cells produced according to the present invention are either a) cells which in their genome has integrated at least two copies of a DNA construct comprising the structure IR(1)-P-IR(2), the structure further comprising a recombination target sequence R
20 or the common homologous recombination sequence R'/R'' inbetween IR(1) and IR(2), which structure is free from any gene encoding an undesirable, e.g. antibiotic resistance marker.

Of particular interest is a cell which in its genome (also outside the structure IR(1)-P-IR(2) further comprising a DNA
25 sequence R or R'/R'') is free from a gene encoding an antibiotic resistance marker or another undesired marker type. The presence of such genes normally follows when multicopy strains are constructed by conventional methods, including transposition when based on transposition delivery vectors known in
30 the art.

Although the cell of the invention is preferably free from any introduced selectable marker it may contain a gene encoding a harmless (i.e. not undesirable) selectable marker such as a a

growth-inhibiting marker or an antibiotic-resistance marker.
These types of markers are discussed in detail above in the
section entitled "Selection marker".

The cell of the invention may be of any gram-positive bacterial
5 species, in particular a cell of a <u>Bacillus</u> sp. or a <u>Lactobacillus</u> sp. Examples of suitable cells of <u>Bacillus</u> may be
selected from the group consisting of <u>Bacillus subtilis</u>,
<u>Bacillus licheniformis</u>, <u>Bacillus lentus</u>, <u>Bacillus brevis</u>,
<u>Bacillus stearothermophilus</u>, <u>Bacillus alkalophilus</u>, <u>Bacillus</u>
10 <u>amyloliquefaciens</u>, <u>Bacillus coagulans</u>, <u>Bacillus circulans</u>,
<u>Bacillus lautus</u>, <u>Bacillus megaterium</u>, <u>Bacillus thuringiensis</u>.
In connection with the use of <u>B. lentus</u> it may be noted that
there has been no prior disclosure or indication to the fact
that transposition can work in cells of of said species.

15 As to the DNA sequence of interest present in the cell of the
invention reference is made to the section "DNA sequence of
interest" discussed above.

Finally, it will be understood that under circumstances where
there is no desire of constructing a marker-free, single or
20 multicopy strain M2 need not be eliminated after having served
the purpose of identifying cells which in their genome comprise
one or more copies of the DNA sequence of interest. In such
cases transposition serves as a convenient method of introducing at least one and preferably multiple copies of a DNA
25 sequence of interest into the genome of a cell.

<u>Production of a polypeptide of interest</u>

The cell of the invention comprising more than one copy of the
structure IR(1)-P-IR(2) further comprising a DNA sequence R or
R'/R'' inbetween IR(1) and IR(2) integrated into its genome or
30 a cell prepared by a method of the invention as described above
may suitably be used in a method for producing a polypeptide of
interest which is encoded by a DNA sequence P of interest.

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The method comprises cultivating the cells in question in a suitable nutrient medium under conditions permitting the expression of the polypeptide, after which the resulting polypeptide is recovered from the culture.

5 The medium used to culture the cells may be any conventional medium suitable for growing the cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of
10 the American Type Culture Collection). The polypeptide produced by the cells may then be recovered from the culture medium by conventional procedures including separating the cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by
15 means of a salt, e.g. ammonium sulphate, purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, gelfiltration chromatography, affinity chromatography, or the like, dependent on the type of polypeptide in question.

20 The polypeptide is preferably a translocated polypeptide, in particular a secreted polypeptide such as a secreted enzyme. Alternatively, the translocated polypeptide is PrsA. Specific examples of polypeptides which may be produced in accordance with this aspect of the invention are given above in the
25 section entitled "The DNA sequence of interest".

Conclusion

From the above description of the invention it will be apparent that the present invention is novel and inventive over the prior art for one or more of the following reasons:

30 1) transposons are used for insertion of more than one copy and a fixed, predetermined number of copies of a gene encoding a product of interest,

2) the transposed DNA contains elements that allow site-speci-
fic deletion of parts of the transposed DNA,
3) the resulting strains contain transposed DNA, but have no
selection marker genes within the transposed DNA, and/or
5 4) transposons are used in an iterative process, made possible
by the combination with a method to specifically delete marker
genes.

From an industrial point of view, bacterial strains constructed
in accordance with the invention may have the triple advantage
10 of being

    i)   high-yielding due to the presence of multiple gene copies,
    ii) Genetically extremely stable, as the multiple copies are
    not tandem repeats but can be scattered over the chromosome,
    and
15 iii) Marker free, making them more "environmentally friendly"
    than traditional recombinant production strains containing
    antibiotic resistance genes.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is further described in the following examples
20 with reference to the appended drawings, in which the following
abbreviations are used:

"bla" indicates the beta-lactamase gene, encoding ampicillin
resistance, from pUC19.
"erm" indicates the erythromycin resistance gene of pE194.
25 "cat" indicates the chloramphenicol resistance gene derived
from pC194.
"IR" indicates the transposon inverted repeat sequence derived
from Tn10.
"Tn ase" indicates the transposase from Tn10, modified to be
30 expressed in Bacillus.
"PamyQ-sav" indicates the Savinase gene, expressed from the
promoter for the B. amyloliquefaciens alpha-amylase gene.

"oriT(pUB110)" indicates the cis-acting sequence of pUB110
necessary for mobilization.

"ermR" indicates the erythromycin resistance gene derived from
pAMβ1.

5 "repE" indicates the replication protein gene of pAMβ1.

"resB" indicates the resolvase gene of pAMβ1.

"topB" indicates the topoisomerase gene of pAMβ1.

"amy'" indicates the 5´end of the B. subtilis amylase gene.

"lacZ" indicates the beta-galactosidase gene from E. coli.

10 "res" indicates the target site for the resolvase from pAMβ1.

"spc" indicates the spectinomycin resistance gene from Tn554.

"'amy" indicates the 3' end of the B. subtilis amylase gene.

"+ ori pUB110" indicates the replication origin of pUB110 (the
nick site).

15 "rep" indicates the replication protein gene of pUB110.

"kan" indicates the kanamycin resistance gene of pUB110.

"PamyL" indicates the promoter of the B. licheniformis alpha-
amylase gene.

"kan'" indicates the 5´end of the kanamycin resistance gene of
20 pUB110.

"'amyL" indicates the 3´end of the B. licheniformis alpha-amy-
lase gene.

"repF" indicates the replication protein gene of pE194.

"Plac" indicates the beta-galactosidase promoter on pUC19.

25 "amyL" indicates the alpha-amylase gene from B. licheniformis.

Fig. 1 is a restriction map of plasmid pMOL553;

Fig. 2 is a restriction map of plasmid pSJ3282;

Fig. 3 is a restriction map of plasmid pWT;

Fig. 4 is a restriction map of plasmid pMAP29;

30 Fig. 5 is a restriction map of plasmid pSJ3157;

Fig. 6 is a restriction map of plasmid pSJ2739;

Fig. 7 is a restriction map of plasmids pSJ3279-pSJ3281;

Fig. 8 is a schematic representation of the oligonucleotide
primers and amplified DNA fragments used for the construction
35 of the res-cat-res-IR segment eventually incorporated into
plasmids pSJ3389-pSJ3390;

Fig. 9 is a restriction map of plasmids pSJ3372-pSJ3376;

Fig. 10 is a restriction map of plasmids pSJ3389-pSJ3390;

Fig. 11A shows the result of a Mancini immunodiffusion assay for Savinase;

5 Fig. 11B shows the result of a Mancini immunodiffusion assay for Savinase;

Fig. 12A is a Southern blot, using DNA from the strains indicated;

Fig. 12B is a Southern blot, using DNA from the strains as in 10 Fig. 12A;

Fig. 13 is a restriction map of plasmid pSJ3216;

Fig. 14 is a restriction map of plasmid pSJ3318;

Fig. 15 is a restriction map of plasmids pSJ3328-pSJ3329;

Fig. 16 is a restriction map of plasmids pSJ3326-pSJ3327;

15 Fig. 17 is a restriction map of plasmids pSJ3341-pSJ3342;

Fig. 18 is a restriction map of plasmids pSJ3358-pSJ3359;

Fig. 19 is a restriction map of plasmids pSJ3354-pSJ3355;

Fig. 20 is a restriction map of plasmids pSJ3444-pSJ3445;

Fig. 21 is a restriction map of plasmid pSJ3475;

20 Fig. 22 is a restriction map of plasmid pSJ3476;

Fig. 23 is a restriction map of plasmids pSJ3316-pSJ3317;

Fig. 24 is a restriction map of plasmids pSJ3339-pSJ3340;

Fig. 25 is a restriction map of plasmids pSJ3356-pSJ3357;

Fig. 26 is a restriction map of plasmid pSJ3385;

25 Fig. 27 is a restriction map of plasmids pSJ3459-pSJ3460;

Fig. 28 is a restriction map of plasmids pSJ3586-pSJ3587;

Fig. 29 is a restriction map of plasmids pSJ3524-psJ3527.

The invention is further illustrated in the following examples which are not in any way intended to limit the scope of the 30 invention as claimed.

MATERIALS AND METHODS

In vitro DNA work, transformation of bacterial strains etc. were performed using standard methods of molecular biology (Maniatis, T., Fritsch, E. F., Sambrook, J. "Molecular Cloning.

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A laboratory manual". Cold Spring Harbor Laboratories, 1982; Ausubel, F. M., et al. (eds.) "Current Protocols in Molecular Biology". John Wiley and Sons, 1995; Harwood, C. R., and Cutting, S. M. (eds.) "Molecular Biological Methods for 5 Bacillus". John Wiley and Sons, 1990). Enzymes for DNA manipulations were used according to the specifications of the suppliers.

Media used (TY, BPX and LB agar) have been described in EP 0 506 780. LBPSG agar is LB agar supplemented with phosphate 10 (0.01 M K_3PO_4), glucose (0.4 %), and starch (0.5 %)

EXAMPLE 1
Construction of a transposon delivery vector for transposition of a Savinase® gene

Transposon donor plasmid pHV1248 (Petit, M.-A., Bruand, C., 15 Janniere, L., Ehrlich, S. D. (1990) Tn10-derived transposons active in Bacillus subtilis. J. Bacteriol., 172, 6736-6740) was used as starting material. This plasmid contain the pE194 replicon which is thermosensitive for replication, and carry a transposase gene from Tn10 modified to be expressed in Bacillus 20 subtilis, and sufficient sequences from the IS10 elements of Tn10 flanking a chloramphenicol resistance gene (mini-Tn10) to allow transposition of mini-Tn10 into the Bacillus subtilis chromosome. pHV1248 was introduced into E. coli SJ6 (Diderichsen et al., 1990, J. Bacteriol. 172, 4315-4321), selecting 25 ampicillin resistance (100 μg/ml), to give SJ1609. Strain SJ1609 was deposited with DSM as DSM10445, as a patent deposit in accordance with the Budapest treaty, on December 22, 1995.

Savinase® is the extracellular, alkaline protease from Bacillus lentus. A vector for the delivery of this gene by transposition 30 is pMOL553. This plasmid was constructed in two steps.

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Step 1.
SOE PCR (Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K.,
Pease, L. R. (1989) Engineering hybrid genes without the use of
restriction enzymes: gene splicing by overlap extension. Gene,
5 77, 61-68) was used to insert a BamHI site upstream of the cat
gene in pHV1248. Two seperate PCR reactions were performed
using pHV1248 as template. In the first PCR reaction was used
primer 1 (LWN5037): CCCACTGGATCCAATTTTCGTTTGTTG and primer 2
(LWN5038: GCAAATTGATCCAAGAGAACCAAC. The underlined bases in
10 primer 1 show the position of the BamHI site. The second PCR
reaction was based on primer 3 (LWN5036): CAACAAACGAAAATTGGATC-
CAGTGGG and primer 4 (LWN5039): GCACATCATCATCATAAGC. Both PCR
reactions were performed by standard procedures using tempera-
tures of 96°C at denaturation, 55°C at annealing and 72°C at
15 the extension step. A total of 20 cycles were performed. Both
fragments were purified from an agarose gel and 500 ng of each
were used for a second 5 cycle PCR reaction: 96°C for 2 min.,
50°C for 5 min and 72°C for 1 min. Primer 2 and primer 4 (100
pmol) were added at 96°C and a third 25 cycle PCR reaction was
20 initiated: 96°C for 30 sec., 55°C for 30 sec. and 72°C for 90
sec. The final PCR fragment of 1330 bp was digested with Hin-
dIII, ligated to HindIII digested pHV1248, and the ligation
mixture transformed into E. coli SJ2 (Diderichsen et al., 1990,
J. Bacteriol. 172, 4315-4321). One transformant kept, MOL612,
25 contained plasmid pMOL610. The position of the BamHI site in
pMOL610 was verified by restriction digest.

Step 2
In this step the entire Savinase® gene was cloned into the Bam-
HI site of pMOL610. The Savinase® gene and a promoter region
30 was amplified from plasmid pSX222 (WO 92/11357) by PCR using
primers with BamHI restriction sites (underlined), primer 5
(LWN5136): CCGGCGGATCCAAGGGGTGATCG and primer 6 (LWN2043): GGG-
GTACTAGTAACCCGGGCCCGGCGTAGAGGATCC ATACACAAA. This Savinase®
gene encodes the wild type Savinase® enzyme, but the DNA
35 sequence is modified to contain a number of restriction enzyme
sites within the Savinase® coding sequence. The PCR reaction

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was performed as follows: 96°C for 30 sec., 55°C for 30 sec., and 72°C for 120 sec. After 20 cycles the PCR fragment was BamHI digested, purified and cloned into the BamHI site of pMOL610. The ligation mixture was transformed into _E. coli_ SJ2, and one transformant kept as MOL553 (SJ2 / pMOL553). The cloning was verified by restriction digests and a distinct protease phenotype in _B. subtilis_ (e.g. in strain DN1885 (Diderichsen et al., 1990, J. Bacteriol. 172, 4315-4321), or protease-deficient derivatives of this strain).

The transposon delivery vector encoding Savinase® thus constructed is pMOL553. The full sequence of this plasmid is given as SEQUENCE ID no. 1, and a restriction map in figure 1.

EXAMPLE 2

Transposition of a Savinase® gene into the _B. lentus_ chromosome, using selection for chloramphenicol resistance

The transposon delivery plasmid pMOL553 was used to insert extra protease genes randomly in the chromosome of _B. lentus_.

A mutant of the alkalophile _Bacillus lentus_ strain C360 (CA 954807) was transformed with the plasmid pMOL553 by protoplast transformation according to a method almost identical to the HCP-1,5 overlayer procedure described by Akamatsu (Akamatsu, T. et al. (1984), Agric. Biol. Chem. 48(3), 651-655). The only major difference was that pH of the HCP-1,5 media and agar was changed to pH 9 to allow the alkalophilic bacilli to regenerate under more suitable conditions. The regeneration plates containing either 10 μg/ml chloramphenicol or 5 μg/ml erythromycin were incubated from 5 to 10 days at 30°C.

After reisolating the transformants on LB9 plates (LB medium containing 0.05 M NaHCO$_3$ buffer pH 9.0) with 2 μg/ml erythromycin, the mini Tn10 transposon carrying the protease gene as well as the cat gene was allowed to transpose according to the method described by Petit et al., 1990. After overnight incuba-

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tion in liquid medium at 46°C, cultures were plated on LB9 +
6µg/ml chloramphenicol at 46°C and colonies screened for eryth-
romycin sensitivity. Eleven erythromycin sensitive, chloramphe-
nicol resistent colonies were selected. A Southern analysis of
the chromosomal DNA isolated from these 11 colonies revealed
that each contained a mini transposon inserted in the chromoso-
me in one copy. 10 strains contained identical insertions,
whereas one contained an insertion in a different position. The
10 identical strains most likely represent siblings from an
early transposition event. None of the transposons had inserted
into the gene coding for the native protease situated in the B.
lentus chromosome.

EXAMPLE 3
Transposition of a Savinase® gene into the Bacillus lentus
chromosome, without selection for chloramphenicol resistance

In an experiment almost identical to that of example 2, a B.
lentus strain with a chromosomal deletion in the alkaline
protease gene was transformed with pMOL553 by the above mentio-
ned protoplast procedure. Throughout this experiment the se-
lection pressure was only for erythromycin resistance, con-
ferred by the vector part of pMOL553. The idea was to isolate
cells that had gained an active protease gene by transposition
of the protease-carrying mini transposon into the chromosome,
by screening for the ability to degrade skimmed milk on an
LB(9) agar plate. The protoplast transformation and reisolation
steps were performed with erythromycin selection only. The
integration period at 30°C allowing the transposon to integrate
was one day in LB(9) medium with 2µg/ml erythromycin. After one
day in LB(9) medium without selection pressure at 46°C the
cells were spread on LB(9) plates with 1% skimmed milk, but no
antibiotics. Among 600 colonies 59 were protease positive.
These 59 colonies were subsequently analyzed with respect to
their antibiotic resistance phenotypes. 19 colonies were re-
sistant to both erythromycin and chloramphenicol indicating
that they still contained pMOL553, while 40 colonies were re-

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sistant to chloramphenicol only, indicating that they had ari-
sen through transposition of the cat and Savinase® genes onto
the chromosome.

Two of the chloramphenicol resistant and erythromycin sensitive
5 colonies were analyzed by Southern hybridization. Both cells
contained two copies of the protease carrying mini transposon
situated in the chromosome at locations different from the
location of the (in this case partly deleted) native protease
gene. All four mini transposon integrations were at different
10 positions in the B. lentus chromosome.

This example thus illustrates the construction of a strain
containing multiple, transposed copies of a sequence of inter-
est, encoding a translocated polypeptide, and illustrates the
isolation of strains containing transposon insertions, without
15 the use of a selectable marker in the strain isolation.

EXAMPLE 4
Construction of a donor strain for conjugative transfer of
plasmids containing oriT from pUB110

Plasmids pLS20 and pBC16 can be transferred by conjugation from
20 B. subtilis strain PSL1 UM13 into various Bacillus recipient
strains (Koehler, T. M. and Thorne, C. B. (1987). Bacillus
subtilis (natto) plasmid pLS20 mediates interspecies plasmid
transfer. J. Bacteriol., 169, 5271-5278).

DN1280 is a derivative of B. subtilis 168, containing a dele-
25 tion in the dal gene (Diderichsen, B. (1986). A genetic system
for stabilization of cloned genes in Bacillus subtilis, p35-46.
In A. T. Ganesan and J. A. Hoch (eds.),Bacillus molecular ge-
netics and biotechnology applications. Academic Press, Inc.,
New York). DN1280 was rendered competent and transformed with
30 plasmid pHV1248, selecting for erythromycin resistance (5
µg/ml) at 30°C. The resulting strain was used as recipient in
conjugation with PSL1 UM13. Both strains, taken from plates

incubated overnight, were mixed on an LBPSG plate (an LB plate supplemented with phosphate (0.01 M K_3PO_4), glucose (0.4 %), and starch (0.5 %)) with D-alanine (100 µg/ml), and incubated for 5 hours at 30°C. The plate was then replicated onto an LBPSG
5 plate as above, but in addition containing erythromycin (5 µg/ml) and tetracycline (5 µg/ml). Single colonies appearing on the replica plate was assayed for their ability to transfer pBC16 into B. subtilis DN1885. Conjugation was performed by mixing of the strains on LBPSG plates as above and incubation
10 for 5 hours at 30°C. Replication was to LBPSG plates with tetracycline (5 µg/ml), but without D-alanine.

The omission of D-alanine effectively counterselects the dal- donor strain. A few of the colonies assayed were able to transfer the Tet[R] marker into DN1885. This indicates that these
15 colonies all harbour pLS20 in addition to pBC16. One such colony was propagated at 50°C in liquid TY medium containing tetracycline (5 µg/ml) and D-alanine (100 µg/ml), subsequently plated on LB containing tetracycline (5 µg/ml) and D-alanine (100 µg/ml), and replica plated onto LB containing D-alanine
20 (100 µg/ml) and erythromycin (5 µg/ml) or chloramphenicol (6 µg/ml), respectively. An tetracycline resistant, erythromycin and chloramphenicol sensitive isolate was kept as PP289-5. This strain, which is dal- and contains pLS20 and pBC16, can serve as a conjugation donor strain that allows the transfer of
25 plasmids containing the pUB110 oriT into various recipient strains.

EXAMPLE 5
Construction of a mobilizable transposon delivery vector for transposition of a Savinase® gene

30 Mobilization of plasmid pUB110 by pLS20 or its derivatives has been described and analyzed in some details (Koehler, T. M. and Thorne, C. B. (1987). Bacillus subtilis (natto) plasmid pLS20 mediates interspecies plasmid transfer. J. Bacteriol., 169, 5271-5278; Selinger, L. B., McGregor, N. F., Khachatourians, G.

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G. and Hynes, M. F. (1990). Mobilization of closely related plasmids pUB110 and pBC16 by Bacillus plasmid pXO503 requires trans-acting open reading frame β. J. Bacteriol., 172, 3290-3297). In this invention elements from these plasmids were used to mobilize transposon delivery vectors.

Mobilization of pUB110 is dependent on a cis acting region (oriT) located 5' to orfβ (Selinger et al.,1990). A 555 bp segment from pUB110, extending from pos. 1020 to pos. 1575 in the pUB110 sequence (McKenzie, T., Hoshino, T., Tanaka, T., Sueoka, N. (1986) The nucleotide sequence of pUB110: some salient features in relation to replication and its regulation. Plasmid 15, 93-103), was PCR amplified using primers LWN5232 and LWN5233.
LWN5232:
5'-GTCGGAGCTCATTATTAATCTGTTCAGCAATCGGGC-3'

LWN5233:
5'-GTCGGAGCTCTGCCTTTTAGTCCAGCTGATTTCAC-3'

The amplified fragment was digested with SacI and initially cloned into the SacI site of an E. coli plasmid (a pUC19 derivative). The fragment was subsequently excised again using SacI, and cloned into the unique SacI site in the previously described plasmid pMOL553. The ligation mixture was transformed into E. coli SJ2 selecting for ampicillin resistance (100 μg/ml).
Plasmids were prepared from the pooled transformants, and the plasmid mixture transformed into PP289-5, selecting resistance to tetracycline (5 μg/ml), chloramphenicol (6 μg/ml) and erythromycin (5 μg/ml) on LBPSG plates containing D-alanine (100 μg/ml). The transformants were again pooled, and the pool used to transfer the oriT-derivative of pMOL553 into DN1885 by conjugation, using the method described above in EXAMPLE 4. Finally, the identity of the plasmids in the transconjugants was verified by restriction mapping, and a strain containing a

correct plasmid was kept, as SJ3282 (DN1885/pSJ3282 (=pMOL553-
oriT; Fig. 2)).

EXAMPLE 6
Construction of a mobilizable plasmid expressing the pAMβ1
5 resolvase

Plasmids pWT and pMAP29 were used as starting material for the
following experiments. pWT is a high copy number pAMβ1 deriva-
tive with the restriction map given in fig. 3. It was transfor-
med into B. subtilis DN1885, and a transformant kept as strain
10 SJ3008. This strain was deposited with the DSM as a patent
deposit in accordance with the Budapest treaty, as DSM10444 on
December 22, 1995. pMAP29 contains two directly repeated
resolution sites (res) flanking a spectinomycin resistance
determinant, and allows insertion of the res/spc/res structure
15 in the amy locus of B. subtilis. It has the map shown in fig.
4. It is kept in an E. coli strain as SJ3007. This strain was
deposited with the DSM as a patent deposit in accordance with
the Budapest treaty, as DSM10446 on December 22, 1995.

pMAP29 was digested with ScaI, and transformed into competent
20 DN1885 selecting spectinomycin resistance (60 μg/ml). One such
transformant was kept as SJ3109. SJ3109 was rendered competent,
and maintained its spectinomycin resistance as competent cells.
pWT, prepared from SJ3008, was transformed into SJ3109 selec-
ting erythromycin resistance (5 μg/ml) at 30 °C. Of 24 trans-
25 formant colonies tested, two were spectinomycin sensitive.
5 spectinomycin resistant transformants were propagated in TY
medium with erythromycin (2 μg/ml) for 2 days at 30 °C. Replica
plating from these cultures revealed only spectinomycin sensi-
tive cells in three, but still spectinomycin resistant cells in
30 the other two. Plasmid pWT was easily cured from the cells by
restreaking two times at 50 °C.

Thus, resolvase as expressed from pWT easily excises the spec-
tinomycin resistance gene from the chromosome of SJ3109.

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The sequence of the pAMβ1 resolvase gene is known, as the re-
solvase is encoded by ORF H as published by Swinfield et al.
(Swinfield, T.-J., Janniere, L., Ehrlich, S. D., Minton, N. P.
(1991). Characterization of a region of the <u>Enterococcus faeca-</u>
5 <u>lis</u> plasmid pAMβ1 which enhances the segregational stability of
pAMβ1-derived cloning vectors in <u>Bacillus subtilis</u>. Plasmid 26,
209-221).
The gene was amplified by a PCR reaction, using as template pWT
and the following primers:

10   BamHI   PstI <   pos. 5102-5129   >
LWN7839:   5'-GACGGGATCCCTGCAGTATCCAATTTATTTTTTTCTTAACAAGG-3'

                EcoRI HindIII< pos. 5820-5797   >
LWN7840:   5'-GACGGAATTCAAAGCTTAAAGCACTTGCATAGGCTAATGCC-3'

15 where the sequence numbering is taken from the above reference.

The amplified DNA fragment was digested with restriction en-
zymes PstI and HindIII, and subsequently ligated to the 4.1 kb
PstI-HindIII fragment isolated from pDN1981 (Jørgensen, P. L.,
Hansen, C. K., Poulsen, G. B., Diderichsen, B. (1990). <u>In vivo</u>
20 genetic engineering: homologous recombination as a tool for
plasmid construction. Gene 96, 37-41) and transformed into <u>B.</u>
<u>subtilis</u> DN1885 selecting kanamycin resistance (10 μg/ml). Two
transformants were kept, SJ3157 containing pSJ3157 (fig. 5),
and SJ3158 containing pSJ3158 (the PstI site in pSJ3158 was
25 destroyed in the cloning, otherwise the plasmids are identi-
cal). pSJ3157 and pSJ3158 are examples of the plasmid pAmyLres,
containing the resolvase gene expressed from the <u>B. lichenifor-</u>
<u>mis amyL</u> promoter, on a pUB110 derived vector conferring
kanamycin resistance.
30 These two plasmids and pDN1981 as a control were transformed
into SJ3109. Kanamycin and spectinomycin resistant colonies (5
with each plasmid) were incubated in TY with kanamycin for 3
days at 30 °C, then spread for replica plating: All resulting
colonies containing pSJ3157 or pSJ3158 were spectinomycin sen-

sitive, whereas colonies containing pDN1981 were spectinomycin resistant.

Resolvase expressed from the Termamyl® (amyL) promoter thus works efficiently in promoting recombination between res sites
5 in the Bacillus chromosome.

In a subsequent step, the amyL promoter and resolvase gene was transferred onto a mobilizable, temperature sensitive cloning vector, pSJ2739 (fig. 6). This vector contains the replication functions and erythromycin resistance gene of pE194
10 (Horinouchi, S., and Weisblum, B. (1982). Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide, and streptogramin type B antibiotics. J. Bacteriol., 150, 804-814), oriT of pUB110, part of the kanamycin resistance gene of pUB110, and a segment of
15 the alpha-amylase gene (amyL) from Bacillus licheniformis. Its full sequence is given as SEQUENCE ID no. 2.

Genes expressed from the amyL promoter on e.g. pDN1981 can conveniently be transferred onto pSJ2739 using BglII and e.g. HindIII. This cloning restores the kanamycin resistance gene.

20 pSJ3157 and pSJ3158, respectively, were digested with HindIII and BglII, the 2.3 kb fragments ligated to the 5.4 kb BglII-HindIII fragment of pSJ2739, and the mixture transformed into competent DN1885 selecting kanamycin (10 μg/ml) and erythromycin (5 μg/ml) resistance at 30 °C. Three transformants kept were
25 SJ3279 (pSJ3279; from pSJ3157)
   SJ3280 (pSJ3280; from pSJ3157) and
   SJ3281 (pSJ3281; from pSJ3158).
   Their restriction map is shown in fig. 7.
   These plasmids are examples of the plasmid pAmyLres(ts).

30 Functionality of these plasmids were assayed by transformation of strain SJ3109; Propagation of SJ3109 containing each plasmid

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resulted in the appearance of spectinomycin-sensitive cells, and the plasmids were subsequently easily cured from the cells by growth without antibiotics at 46 °C.

EXAMPLE 7

Construction of a donor strain for conjugative transfer of the resolvase expressing plasmid

The plasmids constructed in EXAMPLE 6 were transformed into competent cells of the conjugation donor strain PP289-5 (dal⁻, pLS20, pBC16) to allow their easy transfer into other Bacillus strains by conjugation. The following strains were kept:

SJ3308 = PP289-5 / pSJ3279
SJ3309 = PP289-5 / pSJ3280
SJ3310 = PP289-5 / pSJ3281

These strains were able to mobilize the resolvase-expressing plasmid into strain SJ3109, using the procedure for conjugation previously described, where it functioned to excise the spectinomycin resistance gene from the chromosome.

EXAMPLE 8

Insertion of resolvase target sites, res, around the cat marker on the mobilizable transposon delivery vector for transposition of a Savinase® gene

The MluI and SacII sites in pMOL553 (see figure 1) are unique. A modified version of the MluI-SacII fragment can therefore be constructed so as to contain res sites on each end of the cat gene, and can subsequently be ligated to MluI+SacII digested pMOL553. The construction can be performed by PCR amplification, using a number of oligonucleotide primers, by the SOE method as outlined in figure 8. The res site has been located in the pAMβ1 sequence (Swinfield, T. J., Oultram, J. D., Thompson, D. E., Brehm, J. K., Minton, N. P. (1990) Physical

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characterization of the replication region of the <u>Streptococcus</u> <u>faecalis</u> plasmid pAMβ1, Gene 87, pp. 79-90) to pos. 4841-4951 (Janniere, L., Gruss, A., Ehrlich, S. D. (1993), Plasmids, pp. 625-644 in Sonenshein, A. L., Hoch, J. A., Losick, R. (Eds.)
5 <u>Bacillus subtilis</u> and other gram-positive bacteria: Biochemistry, Physiology, and Molecular Genetics. American Society for Microbiology, Washington, D.C.). Accordingly, the PCR primers used for the construction as outlined in figure 8 have the sequences given below:

10 LWN7794:

　　　　KpnI　　MluI
　　5'-GACGGGTACCACGCGTTAATCAATAAAAAAACGCTGTGCGGTTAAA-
　　　　　　　　　　　　　　　　　　BamHI<- X17092 pos.4840-4863 -->
　　　GGGCACAGCGTTTTTTTGTGTATGGATCCTTCTATCTTTTATAGGTCATTAG-3'

15 LWN7790:
　　　pMOL553 pos.3942-3920 >< X17092 pos. 4975-4956
　　5'-TATATATTTTAAAAATATCCCACGGTTCTTCAAATATTTCTCC-3'

LWN7789:
　　x17092 POS.4956-4975 >< pMOL553 pos.3920-3943
20 5'-GGAGAAATATTTGAAGAACCGTGGGATATTTTTAAAATATATAT-3'

LWN7788:
　　x17092 pos.4829-4808　>< pMOL553 pos.4795-4773
　　5'-CAAGTGTTCGCTTCGCTCTCACGGAGCTGTAATATAAAAACCTTC-3'

LWN7787:
25　pMOL553 pos.4773-4795　>< X17092 pos.4808-4829
　　5'-GAAGGTTTTTATATTACAGCTCCGTGAGAGCGAAGCGAACACTTG-3'

52

LWN7784:

   pMOL553 pos.4819-4796   >< X17092 pos.4953-4933
5'-CATATGATCAAATGGTTCGGATCTGATTTTCCTCCTCTAATATGC-3'

LWN8197:

5   X17092 pos.4933-4953 >< pMOL553 pos.4796-4819
5'-GCATATTAGAGGAGGAAAATCAGATCCGAACCATTTGATCATATGACAAGATGTG-3'

LWN7791:

       EcoRI SacII
5'-GACGGAATTCCCGCGGTAAATAGCAATAAATTGGC-3'

10 LWN7780:

       KpnI MluI
5'-GACGGGTACCACGCGTTAATC-3'

PCR fragments A, B, C, and D (figure 8) were prepared as fol-
lows, using 10 pmole of each primer in a standard PCR reaction
15 (25 cycles) with Taq polymerase.

A: Primers LWN7794 and LWN7790, template pWT (from SJ3008), an-
nealing temperature 49 °C. The resulting fragment of 230 bp was
purified from an agarose gel.

B: Primers LWN7789 and LWN7788, template pMOL553, annealing
20 temperature 51 °C. The resulting fragment of 900 bp was purified
from an agarose gel.

C: Primers LWN7787 and LWN7784, template pWT (from SJ3008),
annealing temperature 53 °C. The resulting fragment of 180 bp
was purified from an agarose gel.

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D: Primers LWN8197 and LWN7791, template pMOL553, annealing
temperature 55 °C. The resulting fragment of 275 bp was purified
from an agarose gel.

These fragments were assembled in two steps. In a first step,
5 aliquots of purified A and B fragments were mixed and used as
template in a PCR reaction with primers LWN7780 and LWN7788,
with an annealing temperature of 55 °C. The resulting combined
fragment (AB) of 1100 bp was purified from an agarose gel.
In parallel, aliquots of purified fragments C and D were mixed
10 and used as template in a PCR reaction with primers LWN7787 and
LWN7791, with an annealing temperature of 55 °C. The resulting
combined fragment (CD) of 440 bp was purified from an agarose
gel.

In a second step, the two purified combined fragments, AB and
15 CD, were mixed and used as template in a PCR reaction with
primers LWN7780 and LWN7791, with an annealing temperature of
55 °C. The resulting combined fragment (ABCD) of 1500 bp was
purified from an agarose gel.

The purified fragment (ABCD) was digested with EcoRI and KpnI,
20 ligated to EcoRI + KpnI digested pUC19, and the ligation mix-
ture transformed into E. coli SJ2, selecting resistance to
ampicillin (100 μg/ml) and chloramphenicol (6 μg/ml). 5 trans-
formants were kept, as
SJ3372 = SJ2 / pSJ3372
25 SJ3373 = SJ2 / pSJ3373
SJ3374 = SJ2 / pSJ3374
SJ3375 = SJ2 / pSJ3375
SJ3376 = SJ2 / pSJ3376.
The structure of these plasmids was verified by restriction
30 analysis, and by sequencing of the res and IR parts of the
insert using primers LWN8906: 5'-GGTTTTTCGCATGTATTGCG-3' and

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LWN8907: 5'-GTTCATTTGATATGCCTCC-3', on an Applied Biosystems
automatic sequencer.

A restriction map of pSJ3372-76 is given in figure 9. DNA
sequencing revealed, that the res and IR regions on plasmids
5 pSJ3372 and pSJ3376 contained no mutations due to errors in the
PCR amplification.

The final transposon donor plasmid was constructed by ligating
the 1.5 kb SacII-MluI fragment from either plasmid pSJ3372 or
plasmid pSJ3376 to the 9.6 kb SacII-MluI fragment from pSJ3282.
10 The ligation mixtures were transformed into E. coli SJ2 by
electroporation, and cells plated on either ampicillin (100
μg/ml) plus chloramphenicol (6 μg/ml), or erythromycin (5
μg/ml) plus chloramphenicol (6 μg/ml), at 30 ^{0}C, and transfor-
mants restreaked on all three antibiotics. Two transformants
15 were kept:

SJ3389 = SJ2 / pSJ3389 (from pSJ3376) and
SJ3390 = SJ2 / pSJ3390 (from pSJ3372).

The plasmid structures were verified by PCR amplifications and
restriction analysis. The restriction map of pSJ3389-90 is
20 given in figure 10.

EXAMPLE 9
Construction of a donor strain for conjugative transfer of the
transposon delivery vector of example 8

Competent cells of B. subtilis strain PP289-5 were transformed
25 with pSJ3389, selecting resistance to tetracycline (5 μg/ml),
erythromycin (5 μg/ml), and chloramphenicol (6 μg/ml) on LBPSG
plates with D-alanine (100 μg/ml).
Transformants kept are
SJ3503 = PP289-5 / pSJ3389 and
30 SJ3504 = PP289-5 / pSJ3389

55

EXAMPLE 10
Introduction of the transposon delivery vector of example 8
into B. subtilis, and isolation of strains in which trans-
position has occured

5 B. subtilis DN1885 competent cells were transformed with plas-
mids pSJ3389 or pSJ3390, selecting erythromycin (5 µg/ml) and
chloramphenicol (6 µg/ml) resistance at 30 °C. Four transfor-
mants containing each plasmid were kept, all exhibiting a dis-
tinct protease-positive phenotype on plates with skimmed milk:
10 SJ3431 = DN1885/pSJ3389
    SJ3432 = DN1885/pSJ3389
    SJ3433 = DN1885/pSJ3389
    SJ3434 = DN1885/pSJ3389
    SJ3435 = DN1885/pSJ3390
15 SJ3436 = DN1885/pSJ3390
    SJ3437 = DN1885/pSJ3390
    SJ3438 = DN1885/pSJ3390

These strains were inoculated in 10 ml TY medium with 3 µg/ml
chloramphenicol, and shaken at 30 °C overnight. 100 µl was tra-
20 nsferred to new 10 ml TY medium tubes with 3 µg/ml chloramphe-
nicol, and shaken at 50 °C for 4 hours. A dilution series from
each tube was then plated on LBPSG with 6 µg/ml chloramphenicol
and incubated at 50 °C for two days. These plates were then
replica plated onto
25 i) plates with erythromycin (5 µg/ml) and chloramphenicol (6
µg/ml), and ii) plates with chloramphenicol (6 µg/ml) only, and
the plates incubated at 37 °C overnight.
Among the chloramphenicol resistant colonies, only 10-20 % had
retained the erythromycin resistance, indicating the continued
30 presence of the transposon donor plasmid in these cells. One
chloramphenicol resistant, erythromycin sensitive isolate from
each of the 8 strains was kept:
SJ3465 from SJ3431, SJ3466 from SJ3432, SJ3467 from SJ3433,
SJ3468 from SJ3434, SJ3469 from SJ3435, SJ3470 from SJ3436,
35 SJ3471 from SJ3437, and SJ3472 from SJ3438.

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SJ3470 was distinctly red in liquid TY medium.

A control PCR reaction, using primers LWN5136: 5'-CCGGCGGATC-
CAAGGGGTGATCG-3' and LWN5067: 5'-CCAGAACCTGTCAATCCACG-3', with
annealing temperature 55 °C, was performed on these strains. A
fragment of 900 basepairs, as expected for amplification from
the Savinase® construct on the transposable DNA, was obtained,
confirming the presence of the Savinase® gene in the genome of
these strains.

EXAMPLE 11
**Resolvase mediated marker deletion from the strains of example
10**

Strain SJ3309, described in EXAMPLE 7, is able to donate a
resolvase-expressing plasmid to <u>Bacillus</u> recipient strains by
conjugation.

SJ3309 was streaked on LBPSG plates with D-alanine (100 µg/ml),
tetracycline (5 µg/ml) and erythromycin (5 µg/ml), and incuba-
ted at 30 °C overnight.
Each of the strains SJ3465 to SJ3472 were streaked on LBPSG
plates with chloramphenicol (6 µg/ml) and incubated at 37 °C
overnight.

On separate LBPSG plates containing D-alanine (100 µg/ml), each
of the strains SJ3465 to SJ3472 were mixed with strain SJ3309,
and the plates incubated at 30 °C for 5 hours. Cells were then
resuspended in TY medium and plated on LBPSG plates with eryth-
romycin (5 µg/ml) and chloramphenicol (6 µg/ml), or erythromy-
cin only. These plates were incubated at 30 °C for three days.

Transconjugants where the recipient strains were SJ3465 to
SJ3468, showed good growth on erythromycin only, but poor
growth on both chloramphenicol and erythromycin.

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Transconjugants where the recipient strains were SJ3469 to
SJ3472, showed good growth on both types of plates.
Upon reisolation, transconjugants derived from SJ3465 to SJ3468
could only grow on erythromycin, indicating loss of the chlora-
5 mphenicol resistance gene. Transconjugants derived from SJ3469
to SJ3472 could grow on both types of plates.

To remove the resolvase expressing plasmid from the cells,
transconjugants from SJ3465 to SJ3468 were inoculated in TY
medium and incubated at 50 °C with shaking for 5 hours. Aliquots
10 from these cultures were streaked on LBPSG plates, which were
incubated at 50 °C overnight, and subsequently replicated to
LBPSG, LBPSG with chloramphenicol (10 μg/ml), and LBPSG with
erythromycin (5 μg/ml). These plates were incubated at 37 °C
overnight. One erythromycin sensitive, chloramphenicol sensiti-
15 ve isolate from each culture was kept:
SJ3461 derived from SJ3465
SJ3462 derived from SJ3466
SJ3463 derived from SJ3467
SJ3464 derived from SJ3468.

20 Transconjugants from SJ3469 to SJ3472, having retained their
chloramphenicol resistance, were inoculated in TY medium with
2 μg/ml erythromycin, and shaken at 30 °C for 3 days. Aliquots
were plated on LBPSG at 30 °C, and plates replicated to LBPSG,
LBPSG with chloramphenicol (10 μg/ml), and LBPSG with eryth-
25 romycin (5 μg/ml). All colonies were erythromycin resistant,
but 90 % of colonies from SJ3470, and all from the three ot-
hers, were chloramphenicol sensitive, again indicating loss of
the chloramphenicol resistance gene.
The resolvase expressing plasmid was removed from the strains
30 as above. The following strains were kept:
SJ3489 derived from SJ3469
SJ3490 derived from SJ3470
SJ3491 derived from SJ3472.

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The presence of the Savinase® gene was verified by PCR using primers LWN5136 and LWN5067 as described in example 10.

EXAMPLE 12

Reintroduction of the transposon delivery vector of EXAMPLE 8
5 into the marker free strains of EXAMPLE 11, and isolation of strains in which transposition has occured

pSJ3389 was transferred from SJ3503 and SJ3504 into strain SJ3461 by conjugation on plates, as previously described. After incubation of the mixed strains on D-alanine plates, cells were
10 resuspended and plated on LBPSG containing chloramphenicol (6 μg/ml) and erythromycin (5 μg/ml) at 30 °C. Colonies were checked for tetracycline resistance, indicating presence of pBC16. 3 among 30 checked were tetracycline sensitive. These were kept as
15 SJ3486 = SJ3461 / pSJ3389, SJ3487 = SJ3461 / pSJ3389, and SJ3488 = SJ3461 / pSJ3389.

Strains, in which a new copy of the Savinase® gene had been inserted into the genome by transposition, were isolated essentially as previously described, except that BPX was used
20 as the liquid medium.

Strains SJ3486, 3487 and 3488 (B. subtilis DN1885 containing one chromosomal Savinase® gene copy, plus the transposon donor plasmid), and as a control strain SJ3431 (B. subtilis DN1885 containing only the transposon donor plasmid) were inoculated
25 in 10 ml BPX with chloramphenicol (6 μg/ml), and incubated with shaking for 4 days at 30 °C. Aliquots (appr. 100 μl) were transferred to new 10 ml BPX tubes with chloramphenicol (6 μg/ml), and incubated with shaking for 4 hours at 50 °C. Aliquots from these cultures were then spread on LBPSG contai-
30 ning chloramphenicol (6 μg/ml) at 37 °C, and the resulting

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colonies checked for erythromycin resistance by replica plating. All colonies derived from strain SJ3431 were erythromycin sensitive.

All colonies derived from strains SJ3486 and SJ3488 were eryth-
5 romycin resistant.

About half the colonies from strain SJ3487 were erythromycin sensitive. 10 of these were kept as SJ3537 to SJ3546.

EXAMPLE 13
Resolvase mediated marker deletion from the strains of example
10 12

Strains SJ3537, 3539, 3541 and 3546 were chosen for further work. The resolvase expressing plasmid was introduced into these strains by conjugation from strain SJ3309, as described in example 11 for the one-copy strains.
15 All transconjugant colonies, selected on erythromycin plates, were by replica plating found to be chloramphenicol sensitive, except for a few colonies from the SJ3541 recipient. The excision of the chloramphenicol resistance gene, mediated by resolvase, was thus found to be very efficient. About 50 % of
20 the colonies were tetracycline sensitive, indicating absence of pBC16 (which is present as a helper in the donor strain).

One tetracycline sensitive, chloramphenicol sensitive transconjugant colony from each recipient was propagated in TY at 50 °C for 4 hours, then plated on LBPSG at 50 °C, and the resulting
25 colonies checked for absence of the resolvase-expressing plasmid by replica plating to LBPSG plates with erythromycin (5 μg/ml). All were erythromycin sensitive. One coloni, derived from each recipient strain, was kept:

SJ3565 from SJ3537, SJ3566 from SJ3539, SJ3567 from SJ3541, and
30 SJ3568 from SJ3546.

EXAMPLE 14
Visualisation of Savinase® yields from the strains of examples
10-13

Halo formation on plates:

5 Strains DN1885 (the B. subtilis Savinase® negative recipient
strain), SJ3465-SJ3472 (1. transposition, Cam^R, Sav$^+$), SJ3461
(1. transposition Cam^S, Sav$^+$ (from SJ3465)), SJ3537-SJ3546 (2.
transposition (in SJ3461), Cam^R, Sav$^+$), and SJ3565-SJ3568 (2.
transposition, Cam^S, Sav$^+$) were streaked on LBPSG plates
10 containing 1 % skimmed milk. After overnight incubation at 37
°C, differenses in halo formation were observed. DN1885 had
hardly any halo, 1. transposition strains had a distinct halo,
and 2. transposition strains seemed to have a slightly larger
halo than 1. transposition strains. No difference in halo size
15 was observed due to the removal of the cat gene.

Mancini immunodiffusion assay:

Strains were inoculated in 100 ml BPX shake flasks (with addi-
tion of 0.5 ml 1M NaOH), and shaken for 4 days at 30 °C, 300
rpm.
20 Supernatants were subjected to a Mancini immunodiffusion assay
in an agarose gel containing anti-Savinase antibody.
No precipitation zone was seen with DN1885, in accordance with
the absence of the Savinase® gene from this strain. Zones of
somewhat varying size were seen for the 1. transposition
25 strains (SJ3465-3472). Removal of the cat gene did not signifi-
cantly affect this zone (SJ3461). Zones of conspicuously larger
size were seen from the 2. transposition strains (SJ3537-3546),
and removal of the cat gene did not significantly affect these
zones (SJ3565-3568). The immunodiffusion plates are reproduced
30 in figure 11, A and B.

CONCLUSION:

These phenotypical tests thus revealed the results of the
strain constructions: One copy (at least) of the Savinase® gene
could be inserted by transposition, giving rise to Savinase®
5 production. The chloramphenicol resistance gene on the trans-
posed DNA could be removed using resolvase, and the resulting
strain could still produce Savinase® at the original level. One
(or more) further copies of the Savinase® gene could be
inserted into the one copy strain, reusing the same transposon
10 donor plasmid as used for insertion of the first copy. This
resulted in an increased Savinase® yield. The chloramphenicol
resistance gene on the transposed DNA could subsequently be
removed using resolvase again, and the resulting strains
retained their ability to produce increased Savinase® yields.

15 EXAMPLE 15
Southern analysis of strains of examples 10-13

The strains of examples 10-13 were grown overnight in TY
medium, and chromosomal DNA extracted by standard procedures
(phenol/chloroform extractions). The DNA was digested with
20 EcoRI, which cuts within the Savinase® gene part of the
transposable DNA, and in addition with enzymes BglI, PstI and
SacII, which does not cut within the transposable DNA. Several
enzymes were used together in order to obtain DNA fragments of
moderate size, that could be well resolved on the gel. Frag-
25 ments were transferred to Immobilon-N (Millipore) membranes
after electrophoresis by vacuum blotting, and the membrane
probed with biotinylated labeled probes, using the NEBlot
Phototope Kit and Phototope Detection Kit from New England
Biolabs. The same membrane was used with several sets of
30 probes. First, a probe recognizing the upstream half of the
transposable DNA was used, i.e. the region between the IR at
around pos. 2000 and the EcoRI site at around pos. 3600 in
pSJ3389. The probe was made by PCR amplification from pMOL553,
using as primers LWN5136 and LWN5067. This probe is expected to

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recognize one fragment derived from each transposon insertion, and this fragment should not be affected by the subsequent action of the resolvase. This is exactly what is observed from figure 12A. Strains SJ3465 to SJ3472 were isolated following 5 the first transposition round, and contain both the cat and the savinase gene. As they resulted from different transposition events, different fragment sizes are observed. Strain SJ3461 was isolated from SJ3465, and contain only the savinase gene. The same hybridizing fragment is observed in this strain (2.0 10 kb). Strains SJ3537-3546 were isolated following the second transposition round. They contain the same fragment as SJ3461 (2.0 kb), but has acquired an additional hybridizing fragment of 1.8 kb. The same two hybridizing fragments are observed in strains SJ3565-3568, in which the resolvase gene was used to 15 delete the cat gene (DNA from SJ3565 was degraded by nucleases).

The membrane was subsequently stripped for the probe, and rehybridized with two probes. One was the same as before. The other was the PCR fragment containing res-cat-res-IR, called 20 ABCD in example 8. The first probe should recognize the same fragments as before. The new probe should recognize a new fragment, and the size of this fragment should become reduced by the resolvase mediated deletion of the cat gene. This is exactly what is observed from figure 12B. The new fragment, of 25 about 3.7 kb in strain SJ3465, is reduced to about 2.7 kb in strain SJ3461. Strains SJ3537-46 contain an additional fragment of 3.3 kb, which is reduced to 2.3 kb in strains SJ3566-68.

Fragments were visible at higher positions on the southern blot – these were due to incomplete digestion of the chromosomal DNA 30 with one or more of the enzymes.
    Also, the fragments containing the res-cat-res-IR part of the transposon are actually visible on figure 12A. This is caused by the presence of some plasmid pMOL553 in the material used for labelling (The PCR fragment was not gel purified).

EXAMPLE 16

Construction of a mobilizable transposon delivery vector
containing a res-spc-res cassette

A) Amplification of the spc resistance gene.

5 The spectinomycin resistance gene was obtained from plasmid
pMAP29 (figure 4) on a 1.2 kb SalI-BamHI fragment. This
fragment was purified from an agarose gel, and ligated to XhoI
+ BamHI digested pDN3000 (Diderichsen et al., 1990, J. Bac-
teriol. 172, 4315-4321), to give plasmid pSJ3216 (figure 13).
10 Ligation mixtures were introduced into E. coli SJ2 by elec-
troporation, and selection was for ampicillin (100 µg/ml) and
spectinomycin (60 µg/ml) resistance.

The spc gene was PCR amplified from pSJ3216 using primers
LWN8524: 5'-GACTGAATTCGGATCCACGCGTATAATAAAGAATAATTATTAATCTGTAG-
15 3', and LWN8528: 5'-GACTAAGCTTGAGCTCCACTAATATTAATAAACTATCGAAGG-
3'; Annealing temperature was 50 °C. The fragment was purified
on an agarose gel, digested with EcoRI and HindIII, and ligated
to EcoRI + HindIII digested pUC19. E. coli SJ2 was transformed
by electroporation, selecting ampicillin resistance (100 µg/ml)
20 on plates with IPTG and X-gal. White colonies were reisolated
on plates with ampicillin (100 µg/ml) and spectinomycin (60
µg/ml). Strains kept are SJ3318 (SJ2/pSJ3318) and SJ3319
(SJ2/pSJ3319). The map of pSJ3318 is given in figure 14.

B) Amplification of the res site with multilinker.
25 Plasmid pWT (figure 3) was used as template in a PCR reaction
with primers LWN8529: 5'-GACTGAATTCCTGCAGGAGCTCAGTGAGAGCGAAGC-
GAACAC-3' and LWN8531: 5'-GACTAAGCTTTGATCAAATGGTTGCGGCCGCGT-
CGACTCTAGACCCGGGTACCAGATCTGGATCCTCGGGTTCTTCAAATATTTCTCC-3'; an-
nealing temperature was 59 °C. The fragment was purified from
30 an agarose gel, digested with EcoRI and HindIII, and ligated to
EcoRI + HindIII digested pUC19. E. coli SJ2 was transformed by
electroporation, selecting ampicillin resistance (100 µg/ml) on
plates with IPTG and X-gal. White colonies were reisolated on
similar plates. Plasmids prepared from selected transformants

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were DNA sequenced using primer LWN7191: 5'-GTTTTCCCAGTCACGAC.
Two plasmids with correct sequences of the insert were kept:
SJ3328 (SJ2/pSJ3328) and SJ3329 (SJ2/pSJ3329) (figure 15).

C) Amplification of the res site.
5 Plasmid pWT was used as template in a PCR reaction with primers
LWN8518:   5'-GACTAAGCTTACGCGTTCGGGTTCTTCAAATATTTCTCC-3'   and
LWN8527:   5'-GACTGAATTCTGATCAAATGGTTCAGTGAGAGCGAAGCGAACAC-3';
annealing temperature was 59 °C. The fragment was purified from
an agarose gel, digested with EcoRI and HindIII, and ligated to
10 EcoRI + HindIII digested pUC19. E. coli SJ2 was transformed by
electroporation, selecting ampicillin resistance (100 μg/ml) on
plates with IPTG and X-gal. White colonies were reisolated on
similar plates. Plasmids prepared from selected transformants
were DNA sequenced using primer LWN7191: 5'-GTTTTCCCAGTCACGAC.
15 Two plasmids with correct sequences of the insert were kept:
SJ3326 (SJ2/pSJ3326) and SJ3327 (SJ2/pSJ3327) (figure 16).

D) Construction of a spc-res fragment.
The 1.1 kb SacI-EcoRI fragment from pSJ3318 was isolated from
an agarose gel, and ligated to SacI + EcoRI digested pSJ3328.
20 The ligation mixture was introduced into E. coli SJ6 by elec-
troporation, and transformants selected on plates with ampi-
cillin (100 μg/ml) and spectinomycin (60 μg/ml). Two transfor-
mants were kept, SJ3341 (SJ6/pSJ3341) and SJ3342 (SJ6/pSJ3342)
(figure 17).

25 E) Construction of a res-spc-res fragment.
The 0.2 kb EcoRI-MluI fragment from pSJ3326 was purified from
an agarose gel, and ligated to EcoRI + MluI digested pSJ3341.
The ligation mixture was introduced into E. coli SJ6 by elec-
troporation, and transformants selected on plates with ampicil-
30 lin (100 μg/ml) and spectinomycin (60 μg/ml). Two transformants
were kept, SJ3358 (SJ6/pSJ3358) and SJ3359 (SJ6/pSJ3359)
(figure 18).

F) Cloning of minitransposon (IR-cat-IR) in pUC.

The minitransposon segment (without the transposase gene) was
excised from pHV1248 on a 1.2 kb HindIII fragment, which was
ligated to HindIII digested pUC19 and used to transform E. coli
5 SJ6 by electroporation to ampicillin (100 μg/ml) and chloramp-
henicol (10 μg/ml) resistance. Two transformants were kept,
SJ3354 (SJ6/pSJ3354) and SJ3355 (SJ6/pSJ3355) (figure 19).

G) Insertion of the res-spc-res segment into minitransposon.

The minitransposon contained BsaBI sites in the IR sequences
10 outside the terminal regions essential for transposition. These
sites were used for insertion of the res-spc-res segment.

The res-spc-res segment was excised from pSJ3358 on a 1.6 kb
HincII-PvuII fragment, purified from an agarose gel, and liga-
ted to the 2.8 kb BsaBI fragment of pSJ3354. The ligation
15 mixture was used to transform, by electroporation, E. coli
strain SJ6 to ampicillin (100 μg/ml) and spectinomycin (60
μg/ml) resistance. Two transformants were kept, SJ3444
(SJ6/pSJ3444) and SJ3445 (SJ6/pSJ3445) (figure 20).

H) Construction of a res-spc-res containing transposon donor
20 plasmid.

The transposable cassette was excised on a 1.85 kb HindIII
fragment from plasmid pSJ3444, which was ligated to the 7.85 kb
HindIII fragment from pSJ3282. The ligation mixture was
introduced into E. coli SJ6 by electroporation, selecting
25 ampicillin (100 μg/ml) and spectinomycin (60 μg/ml) resistance.
Two transformants were kept, with the transposable cassette in
different orientations relative to the vector part: SJ3475
(SJ6/pSJ3475; figure 21) and SJ3476 (SJ6/pSJ3476; figure 22).

The functionality of this transposon donor plasmid was tested.
30 Plasmids pSJ3475 and pSJ3476 were transformed into competent B.
subtilis DN1885 selecting for erythromycin (5 μg/ml) and spec-
tinomycin (60 μg/ml) resistance. Four transformants with each
plasmid were taken through a transposition round: They were

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grown overnight in 10 ml TY with spectinomycin (60 μg/ml). 50
μl was transferred to a new culture of 10 ml TY with spectino-
mycin (60 μg/ml), incubated at 50 °C for 4 hours (resulting in
good growth of pSJ3475 transformants, poor growth of pSJ3476
5 transformants), and plated on LBPSG with spectinomycin (60
μg/ml) at 50 °C. 10 single colonies from each plate was streaked
on duplicate plates with erythromycin (5 μg/ml) and spec-
tinomycin (60 μg/ml), respectively. Most colonies were eryth-
romycin sensitive, indicating proper transposition of the IR-
10 res-spc-res-IR segment and loss of the donor plasmid. 2 Spc^R,
Erm^S colonies derived from each of pSJ3475 and pSJ3476 trans-
formants were used as recipients for conjugation with SJ3309 as
donor, and transconjugants selected at 30°C on plates with kana-
mycin (10 μg/ml) and with or without spectinomycin (60 μg/ml).
15 Good growth was observed on kanamycin plates, but transcon-
jugants from three of the four strains grew poorly on plates
with both antibiotics. Spectinomycin sensitive, kanamycin re-
sistant colonies were in all instances obtained by reisolation
from the kanamycin plates.
20 Thus, the modified transposon functions in B. subtilis, and the
spectinomycin resistance gene can be subsequently deleted using
resolvase.

EXAMPLE 17
Construction of a mobilizable transposon delivery vector
25 containing a res-kan-res cassette

A) Amplification of the kan resistance gene.
The kan gene was PCR amplified from pUB110 using primers
LWN8516: 5'-GACTGAATTCGGATCCACGCGTGAGTAGTTCAACAAACGGGCC-3' and
LWN8517: 5'-GACTAAGCTTGAGCTCCAACATGATTAACAATTATTAGAGG-3'; an-
30 nealing temperature was 59 °C. The fragment was purified from
an agarose gel, digested with EcoRI and HindIII, and ligated to
EcoRI + HindIII digested pUC19. E. coli SJ2 was transformed by

electroporation, selecting ampicillin resistance (100 µg/ml) on
plates with IPTG and X-gal. White colonies were reisolated on
plates with ampicillin (100 µg/ml) and kanamycin (20 µg/ml).
Strains kept are SJ3316 (SJ2/pSJ3316) and SJ3317 (SJ2/pSJ3317)
5 (figure 23).

B) Construction of a kan-res fragment.
The 1.0 kb SacI-EcoRI fragment from pSJ3316 was isolated from
an agarose gel, and ligated to SacI + EcoRI digested pSJ3328.
The ligation mixture was introduced into E. coli SJ6 by elec-
10 troporation, and transformants selected on plates with ampi-
cillin (100 µg/ml) and kanamycin (10 µg/ml). Two transformants
were kept, SJ3339 (SJ6/pSJ3339) and SJ3340 (SJ6/pSJ3340)
(figure 24).

C) Construction of a res-kan-res fragment.
15 The 0.2 kb EcoRI-MluI fragment from pSJ3326 was purified from
an agarose gel, and ligated to EcoRI + MluI digested pSJ3340.
The ligation mixture was introduced into E. coli SJ6 by elec-
troporation, and transformants selected on plates with ampicil-
lin (100 µg/ml) and kanamycin (10 µg/ml). Two transformants
20 were kept, SJ3356 (SJ6/pSJ3356) and SJ3357 (SJ6/pSJ3357)
(figure 25).

D) Construction of a res-kan-res containing transposon donor
plasmid.

i) Cloning of a transposon IR sequence.
25 The "downstream" transposon IR sequence was PCR amplified from
pMOL553 as template, using primers LWN8760: 5'-GACGGAATTCTC-
TAGAGTCGACAGATCCGAACCATTTGATCATATGACAAGATGTG-3' and LWN8761:
5'-GACGGAATTCGCGGCCGCGGTAAATAGCAATAAATTGGCC-3'. The purified
fragment was digested with EcoRI, ligated to EcoRI digested
30 pUC19, and introduced by electroporation into E. coli SJ2,
selecting ampicillin resistance (100 µg/ml) on plates with IPTG

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and X-gal. About 20 white colonies among 500 was obtained.
Plasmid from three was DNA sequenced using as primer LWN4123:
5'-AGCGGATAACAATTTCACACAGGA-3'. Two correct clones were kept as
SJ3385 (SJ2/pSJ3385) (figure 26) and SJ3386 (SJ2/pSJ3386).

5 ii) Combination of one IR with res-kan-res sequence.
The IR sequence was excised from pSJ3385 as a 0.3 kb NotI-XbaI
fragment, ligated to NotI + XbaI digested, phosphatase treated
pSJ3356, and introduced into SJ6 by electroporation, selecting
ampicillin (100 μg/ml) and kanamycin (10 μg/ml) resistance. Two
10 transformants were kept as SJ3459 (SJ6/pSJ3459) and SJ3460 (SJ-
6/pSJ3460) (figure 27).

iii) Construction of the final transposon donor plasmid.
In this step, the spc-res segment from donor plasmid pSJ3476
was replaced with the corresponding kan-res segment from
15 pSJ3459. Thus, this 1.15 kb fragment was excised from pSJ3459
using MluI and BamHI, and the purified fragment ligated to the
8.2 kb MluI-BamHI fragment from pSJ3476. The mixture was
introduced into E. coli SJ6 by electroporation, selecting
ampicillin (100 μg/ml) and kanamycin (10 μg/ml) resistance. Two
20 transformants were kept as SJ3586 (SJ6/pSJ3586) and SJ3587
(SJ6/pSJ3587) (figure 28).

EXAMPLE 18
Use of the transposon delivery vector of EXAMPLE 16 for
transposition of an amylase gene into the B. subtilis genome

25 The B. licheniformis amylase gene (amyL) gene was excised from
pDN1981 as a 3.2 kb BglII-BclI fragment, and ligated to BamHI
digested pSJ3476. The ligation mixture was transformed into B.
subtilis DN1885, selecting erythromycin resistance (5 μg/ml) at
30 °C. Four amylase positive colonies were reisolated on
30 erythromycin (5 μg/ml) and spectinomycin (60 μg/ml) plates, and

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kept as SJ3524 (DN1885/pSJ3524), SJ3525 (DN1885/pSJ3525), SJ3526 (DN1885/pSJ3526), and SJ3527 (DN1885/pSJ3527) (figure 29).

Strains SJ3524-SJ3527 were tested for transposition. Each was
5 inoculated in LB with erythromycin (5 μg/ml) and spectinomycin (60 μg/ml), incubated overnight at 30 °C, and aliquots trans- ferred to LB with spectinomycin (60 μg/ml). These cultures were shaken at 50 °C for 5 hours, then plated on spectinomycin plates (60 μg/ml) and incubated at 30 °C overnight. Plates from SJ3524-
10 3526 were almost overgrown; 8 colonies from each were tested and found erythromycin resistant. The plate from SJ3527 was replica plated, and several erythromycin sensitive, spec- tinomycin resistant and amylase postivive colonies were found. One was kept as SJ3549.

15 EXAMPLE 19
Resolvase mediated marker deletion from the strain of EXAMPLE 18

The resolvase-expressing plasmid was introduced into SJ3549 by conjugation from SJ3309 as previously described. Transconju-
20 gants were selected on kanamycin (10 μg/ml) and spectinomycin (60 μg/ml), and reisolated on kanamycin (10 μg/ml) with or without spectinomycin (60 μg/ml). Growth was poor on plates with both antibiotics, good on kanamycin plates. 10 colonies from kanamycin plates were reisolated on LBPSG, and subsequent-
25 ly replica plated. 8 spectinomycin sensitive, kanamycin re- sistant kolonies thus isolated were inoculated in TY and in- cubated at 50 °C overnight, then plated on LBPSG, and these pla- tes replicated after overnight incubation at 37 °C. All co- lonies, except some from culture 1, were kanamycin sensitive.
30 Reisolation confirmed sensitivity to kanamycin (10 μg/ml) and erythromycin (5 μg/ml). Three strains were kept:
SJ3558 (Spc^S, Erm^S, Kan^S, Amy$^+$)
SJ3559 (Spc^S, Erm^S, Kan^S, Amy$^+$)

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SJ3560 (Spc^s, Erm^s, Kan^s, Amy$^+$)

The three strains were tetracycline sensitive, indicating ab-
sence of pBC16, which is present in the conjugation donor str-
ain.

5 EXAMPLE 20
Construction of a donor strain for conjugative transfer of the
transposon delivery vector of example 18

Plasmids pSJ3524 and pSJ3526 were transformed into competent
cells of a B. subtilis donor strain (dal$^-$, pX0503 (erm^R), pBC16
10 (tet^R)) for conjugation, plated on LBPSG with erythromycin (5
µg/ml), tetracycline (5 µg/ml), spectinomycin (60 µg/ml) and D-
alanine (100 µg/ml) at 30 °C, and one strain containing each
plasmid was kept: SJ3547, containing pSJ3524, and SJ3548,
containing pSJ3526.

15 EXAMPLE 21
Reintroduction of the transposon delivery vector of EXAMPLE 18
into the marker free strains of example 19, and isolation of
strains in which transposition has occured

Plasmid pSJ3524 was transferred into SJ3559, a marker free amyL
20 expressing strain constructed in example 19, by conjugation
from SJ3547 as previously described. One tetracycline sensitive
transconjugant was kept as SJ3592. This strain was incubated
with shaking in TY with spectinomycin (60 µg/ml) at 30 °C
overnight, diluted 100 times into a new TY culture with spec-
25 tinomycin (60 µg/ml), and incubated with shaking for 3 hours at
30 °C. It was then diluted 50 times into a new TY culture with
spectinomycin (60 µg/ml), incubated with shaking for 3 hours at
50 °C, and subsequently plated on LBPSG with spectinomycin (60
µg/ml) at 50 °C. Almost all of the resulting colonies were found

to be erythromycin sensitive by replica plating. Four were kept, as SJ3626-SJ3629.

In a similar experiment, pSJ3524 was transferred into SJ3560 by conjugation from SJ3547, and two tetracycline sensitive
5 transconjugants kept as SJ3593 and SJ3594. These were incubated with shaking in TY with spectinomycin (60 µg/ml) at 30 °C overnight, diluted 100 times into a new TY culture with spectinomycin (60 µg/ml), incubated with shaking for 6 hours at 50 °C, and plated on LBPSG with spectinomycin (60 µg/ml) at 50
10 °C. By replica plating, one spectinomycin resistant and erythromycin sensitive colony was found from SJ3593 (kept as SJ3599), and two such colonies were found from SJ3594 (kept as SJ3600 and SJ3601).

EXAMPLE 22
15 Resolvase mediated marker deletion from the strains of example 21

Strains SJ3626-3629 were used as recipients to recieve the resolvase expressing plasmid by conjugation from strain SJ3309. Transconjugants were selected on LBPSG with kanamycin (10
20 µg/ml). Colonies from these plates were replicated to kanamycin (10 µg/ml) and spectinomycin (60 µg/ml), and all were found to be spectinomycin sensitive. 4 colonies derived from each recipient strain were transferred three consecutive times through overnight TY cultures at 30 °C, plated on LBPSG at 30
25 °C, and replica plated to LBPSG with erythromycin. Sensitive colonies were obtained in each case, and these were subsequently checked for kanamycin resistance. One erythromycin, kanamycin, and spectinomycin sensitive isolate derived from each recipient was kept: SJ3634 (from SJ3626), SJ3635 (from SJ3627),
30 SJ3636 (from SJ3628), and SJ3637 (from SJ3629).

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Strains from both 1. and 2. transposition round were inoculated in BPX shake flasks and incubated with shaking for 6 days at 37 °C. Alpha-amylase activity was determined using the Phadebas Amylase Test from Kabi Pharmacia. The results are given here in 5 relative, arbitrary units:

	Units/ml
B. subtilis DN1885 host strain	0.4
(in triplicate)	0.26
	0.25
10 SJ3549 (1. transposition, Spc^R)	2.45
(in duplicate)	2.42
SJ3559 (1. transposition, Spc^S)	2.4
(in triplicate)	2.16
	2.35
15 SJ3626)	3.28
SJ3627) (2. transposition,	3.6
SJ3628) Spc^R)	3.25
SJ3629)	3.14
SJ3634)	3.21
20 SJ3635) (2. transposition,	3.3
SJ3636) Spc^S)	3.38
SJ3637)	3.1

It is evident that a certain production of alpha-amylase is obtained from the 1. transposition strain, this level is unaf-25 fected by the subsequent deletion of the spectinomycin re-sistance gene mediated by resolvase, the level is increased in the 2. transposition strains, and this increased level is main-tained upon deletion of the spectinomycin resistance gene me-diated by resolvase.

In a similar experiment, strains SJ3599-3601 were used as recipients to recieve the resolvase expressing plasmid by conjugation from strain SJ3309. Transconjugants were selected on LBPSG with kanamycin (10 μg/ml). Colonies from these plates

5 were replicated to kanamycin (10 μg/ml), spectinomycin (60 μg/ml), and tetracycline (5 μg/ml). From each recipient, kanamycin resistant but spectinomycin and tetracycline sensitive colonies could be isolated.

The resolvase expressing plasmid, conferring resistance to both

10 kanamycin and erythromycin, could subsequently be cured from the cells by a few successive transfers of strains in liquid TY medium at 30 °C (without antibiotics), and plasmid free cells isolated by replica plating to plates with erythromycin (5 μg/ml). One such erythromycin sensitive strain isolated from a

15 kanamycin resistant transconjugant of SJ3600 was kept as SJ36-40.

EXAMPLE 23
Introduction of the transposon delivery vector of example 18
into the marker free strains of example 13, and isolation of

20 strains in which transposition has occured

In this and the following example, the tools developed in previous examples are combined to create a Bacillus subtilis strain, free of antibiotic resistance markers, containing two copies of the Savinase® gene and one copy of the B. lichenifor-

25 mis amylase gene in its genome.

Strains SJ3565 and SJ3566, each containing two copies of the Savinase® gene, were used as recipients in a conjugation with strain SJ3547. Transconjugants were selected on LBPSG with spectinomycin (60 μg/ml) and erythromycin (5 μg/ml). All were

30 amylase-positive and protease-positive. About half were tetracycline sensitive, indicating absence of pBC16. Two tetracycline sensitive colonies derived from each recipient

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were kept: SJ3595 and SJ3596, from SJ3565, and SJ3597 and
SJ3598, from SJ3566.

These strains were incubated with shaking in TY with spec-
tinomycin (60 µg/ml) at 30 °C overnight, diluted 100 times into
5 a new TY culture with spectinomycin (60 µg/ml), incubated with
shaking for 6 hours at 50 °C, and plated on LBPSG with spec-
tinomycin (60 µg/ml) at 50 °C. These plates were subsequently
replicated to plates with either spectinomycin (60 µg/ml) or
erythromycin (5 µg/ml). No erythromycin sensitive colonies were
10 obtained from SJ3598, whereas about 90 % of the colonies from
the other three strains were spectinomycin resistant, but
erythromycin sensitive. One such colony from each strain was
kept: SJ3602 (from SJ3595), SJ3603 (from SJ3596), and SJ3604
(from SJ3597).

15 EXAMPLE 24

Resolvase mediated marker deletion from the strains of example
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The resolvase expressing plasmid was transferred from strain
SJ3309 into each of strains SJ3602-SJ3604 by conjugation as
20 previously described. Transconjugants were selected on LBPSG
plates with kanamycin (10 µg/ml) at 30 °C, and replica plated
to plates with either kanamycin (10 µg/ml), spectinomycin (60
µg/ml), or tetracycline (5 µg/ml). Spectinomycin and tetra-
cycline sensitive colonies were obtained derived from each
25 recipient strain.

The resolvase expressing plasmid, conferring resistance to both
kanamycin and erythromycin, could subsequently be cured from
the cells by a few successive transfers of strains in liquid TY
medium at 30 °C (without antibiotics), and plasmid free cells
30 isolated by replica plating to plates with erythromycin (5
µg/ml). One such erythromycin sensitive strain isolated from a
kanamycin resistant transconjugant from each of strains SJ3602-

3604 were kept as SJ3641 (from SJ3602), SJ3642 (from SJ3603)
and SJ3643 (from SJ3604).

EXAMPLE 25
Construction and use of a transposon donor plasmid containing
the transposase gene, together with a marker gene, flanked by
res sequences.

The purpose of this example is to create and use a transposon
donor plasmid, in which transposase and marker together resides
between res sequences. This plasmid is then used to isolate
strains where transposition has taken place, and the donor
plasmid has been lost. The transposed fragment is able to
undergo further transpositions, which can be selected for by
increased antibiotic resistance. When desired strains are
obtained, resistance genes and transposases can be deleted
using resolvase, as described in previous examples.

Previously constructed res-marker-res cassettes (examples 16
and 17) contain a unique MluI site, into which transposase can
be inserted.

(1)   Transposase is PCR amplified from pHV1248, using as
primers
          BamHI MluI   <-pHV1248 125-142->
Tnase1: 5'-TGACGGATCCACGCGTGGCGCACTCCCGTTCTGG-3'
and
          BamHI MluI   <-pHV1248 1550-1531->
Tnase2: 5'-GTACGGATCCACGCGTAAAGGCACCTTTGGTCACGG-3'

The PCR fragment is gel purified, digested with BamHI, and
cloned into pUC19 digested with BamHI. Some clones are DNA
sequenced, and a correct one used in further work.

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(2)   A spectinomycin resistance cassette with transposase
is constructed by cloning of the MluI fragment from (1), above,
into pSJ3444.

(3)   A kanamycin resistance cassette with transposase is
5 constructed in two steps: i) The HindIII fragment from pSJ3586
is cloned into pUC19. ii) The MluI fragment from (1) is cloned
into i).

The HindIII fragments can then be cloned into pSJ3476 or
pSJ3282. The resulting plasmid will have two transposase genes,
10 and clones with these genes in antiparallel orientation can be
used as transposon delivery vectors, into which e.g. B.
licheniformis amylase or Savinase® genes may be inserted.

Vector plasmids with only one transposase gene may be prepared
as follows:

15 (4)   The NheI-XbaI fragment from pSJ3475, containing the coli-
vector part of this plasmid, and the transposase, is deleted.
This plasmid is then used as vector for cloning of the HindIII
fragments containing the res-marker-transposase-res segments
described above.

20 (5)   The ScaI-HindIII fragment from pSJ3282 is replaced by
the ScaI-HindIII fragment from pUC18 (this deletes the entire
transposon part). Subsequently this new plasmid is used for
cloning of HindIII fragments, as above.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT:
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 10   (H) TELEFAX: +45 44 49 32 56
        (I) TELEX: 37304

    (ii) TITLE OF INVENTION: TITLE

    (iii) NUMBER OF SEQUENCES: 36

 15 (iv) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

 20 (2) INFORMATION FOR SEQ ID NO: 1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 10216 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
 25   (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid
        (A) DESCRIPTION: /desc = "pMOL553"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

    TTCTCATGTT TGACAGCTTA TCATCGACTG CACGGTGCAC CAATGCTTCT GGCGTCAGGC   60

 30 AGCCATCGGA AGCTGTGGTA TGGCTGTGCA GGTCGTAAAT CACTGCATAA TTCGTGTCGC   120

    TCAAGGCGCA CTCCCGTTCT GGATAATGTT TTTTGCGCCG ACATCATAAC GGTTCTGGCA   180

    AATATTCTGA AATGAGCTGT TGACAATTAA TCATCGGCTC GTATAATGTG TGGAATTGTG   240

    AGCGGATAAC AATTTCACAC AGGAAACAGG ATCAAATGGT TTCGAATTCA TTAAAGAGGA   300

    GAAATTAACT ATGTGCGAAC TCGATATTTT ACACGACTCT CTTTACCAAT TCTGCCCCGA   360

 35 ATTACACTTA AAACGACTCA ACAGCTTAAC GTTGGCTTGC CACGCATTAC TTGACTGTAA   420

    AACTCTCACT CTTACCGAAC TTGGCCGTAA CCTGCCAACC AAAGCGAGAA CAAAACATAA   480

```
  CATCAAACGA ATCGACCGAT TGTTAGGTAA TCGTCACCTC CACAAAGAGC GACTCGCTGT    540

  ATACCGTTGG CATGCTAGCT TTATCTGTTC GCGCAATACG ATGCCCATTG TACTTGTTGA    600

  CTGGTCTGAT ATTCGTGAGC AAAAACGACT TATGGTATTG CGAGCTTCAG TCGCACTACA    660

  CGGTCGTTCT GTTACTCTTT ATGAGAAAGC GTTCCCGCTT TCAGAGCAAT GTTCAAAGAA    720

5 AGCTCATGAC CAATTTCTAG CCGACCTTGC GAGCATTCTA CCGAGTAACA CCACACCGCT    780

  CATTGTCAGT GATGCTGGCT TTAAAGTGCC ATGGTATAAA TCCGTTGAGA AGCTGGGTTG    840

  GTACTGGTTA AGTCGAGTAA GAGGAAAAGT ACAATATGCA GACCTAGGAG CGGAAAACTG    900

  GAAACCTATC AGCAACTTAC ATGATATGTC ATCTAGTCAC TCAAAGACTT TAGGCTATAA    960

  GAGGCTGACT AAAAGCAATC CAATCTCATG CCAAATTCTA TTGTATAAAT CTCGCTCTAA   1020

10 AGGCCGAAAA AATCAGCGCT CGACACGGAC TCATTGTCAC CACCCGTCAC CTAAAATCTA   1080

  CTCAGCGTCG GCAAAGGAGC CATGGTTCTA GCAACTAACT TACCTGTTGA AATTCGAACA   1140

  CCCAAACAAC TTGTTAATAT CTATTCGAAG CGAATGCAGA TTGAAGAAAC CTTCCGAGAC   1200

  TTGAAAAGTC CTGCCTACGG ACTAGGCCTA CGCCATAGCC GAACGAGCAG CTCAGAGCGT   1260

  TTTGATATCA TGCTGCTAAT CGCCCTGATG CTTCAACTAA CATGTTGGCT TGCGGGCGTT   1320

15 CATGCTCAGA AACAAGGTTG GGACAAGCAC TTCCAGGCTA ACACAGTCAG AAATCGAAAC   1380

  GTACTCTCAA CAGTTCGCTT AGGCATGGAA GTTTTGCGGC ATTCTGGCTA CACAATAACA   1440

  AGGGAAGACT TACTCGTGGC TGCAACCCTA CTAGCTCAAA ATTTATTCAC ACATGGTTAC   1500

  GCTTTGGGGA AATTATGAGG GGATCTTCGA CCGTGACCAA AGGTGCCTTT TATCATCACT   1560

  TTAAAAATAA AAAACAATTA CTCAGTGCCT GTTATAAGCA GCAATTAATT ATGATTGATG   1620

20 CCTACATCAC AACAAAAACT GATTTAACAA ATGGTTGGTC TGCCTTAGAA AGTATATTTG   1680

  AACATTATCT TGATTATATT ATTGATAATA ATAAAAACCT TATCCCTATC CAAGAAGTGA   1740

  TGCCTATCAT TGGTTGGAAT GAACTTGAAA AAATTAGCCT TGAATACATT ACTGGTAAGG   1800

  TAAACGCCAT TGTCAGCAAA TTGATCCAAG AGAACCAACT TAAAGCTTTC CTGACGGAAT   1860

  GTTAATTCTC GTTGACCCTG AGCACTGATG AATCCCCTAA TGATTTTGGT AAAAATCATT   1920

25 AAGTTAAGGT GGATACACAT CTTGTCATAT GATCCCGGAT CTGGGCAATA GTTACCCTTA   1980

  TTATCAAGAT AAGAAAGAAA AGGATTTTTC GCTACGCTCA AATCCTTTAA AAAAACACAA   2040

  AAGACCACAT TTTTTAATGT GGTCTTTATT CTTCAACTAA AGCACCCATT AGTTCAACAA   2100

  ACGAAAATTG GATCCAAGGG GTGATCGGTC GGCGGAAATG AAGGCCTGCG GCGAGTGCGG   2160
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GCCTTCTGTT TTGAGGATTA TAATCAGAGT ATATTGAAAG TTTCGCGATC TTTTCGTATA    2220

ATTGTTTTAG GCATAGTGCA ATCGATTGTT TGAGAAAAGA AGAAGACCAT AAAAATACCT    2280

TGTCTGTCAT CAGACAGGGT ATTTTTTATG CTGTCCAGAC TGTCCGCTGT GTAAAAATAA    2340

GGAATAAAGG GGGGTTGTTA TTATTTTACT GATATGTAAA ATATAATTTG TATAAGAAAA    2400

5   TGAGAGGGAG AGGAAACATG ATTCAAAAAC GAAAGCGGAC AGTTTCGTTC AGACTTGTGC    2460

TTATGTGCAC GCTGTTATTT GTCAGTTTGC CGATTACAAA AACATCAGCC GTAAATGGCA    2520

CGCTGATGCA GTATTTTGAA TGGTATACGC CGAACGACGG CCAGCATTGG AAACGATTGC    2580

AGAATGATGC GGAACATTTA TCGGATTAAC TTAACGTTAA TATTTGTTTC CCAATAGGCA    2640

AATCTTTCTA ACTTTGATAC GTTTAAACTA CCAGCTTGGA CAAGTTGGTA TAAAAATGAG    2700

10  GAGGGAAACC GAATGAAGAA ACCGTTGGGG AAAATTGTCG CAAGCACCGC ACTACTCATT    2760

TCTGTTGCTT TTAGTTCATC GATCGCATCG GCTGCTGAAG AAGCAAAAGA AAAATATTTA    2820

ATTGGCTTTA ATGAGCAGGA AGCTGTCAGT GAGTTTGTAG AACAAGTAGA GGCAAATGAC    2880

GAGGTCGCCA TTCTCTCTGA GGAAGAGGAA GTCGAAATTG AATTGCTTCA TGAATTTGAA    2940

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82

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(2) INFORMATION FOR SEQ ID NO: 2:

25   (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 6169 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

84

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     (ii) MOLECULE TYPE: other nucleic acid
          (A) DESCRIPTION:   /desc = "pSJ2739"

     (vi) ORIGINAL SOURCE:
          (A) ORGANISM: pSJ2739


5    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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     AAATTCCTGC CTTGAAACAC AAAATTGAAC CGATCTTAAA AGCGAGAAAA CAGTATGCGT     180

     ACGGAGCACA GCATGATTAT TTCGACCACC ATGACATTGT CGGCTGGACA AGGGAAGGCG     240

10   ACAGCTCGGT TGCAAATTCA GGTTTGGCGG CATTAATAAC AGACGGACCC GGTGGGGCAA     300

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25 (2) INFORMATION FOR SEQ ID NO: 3:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 27 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
30   (D) TOPOLOGY: linear

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```
        (ii) MOLECULE TYPE: other nucleic acid
             (A) DESCRIPTION:   /desc = "LWN5037"


        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

     CCCACTGGAT CCAATTTTCG TTTGTTG                                    27

5 (2) INFORMATION FOR SEQ ID NO: 4:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 24 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
10           (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid
             (A) DESCRIPTION:   /desc = "LWN5038"


        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

     GCAAATTGAT CCAAGAGAAC CAAC                                       24

15 (2) INFORMATION FOR SEQ ID NO: 5:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 27 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
20           (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid
             (A) DESCRIPTION:   /desc = "LWN5036"


        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

     CAACAAACGA AAATTGGATC CAGTGGG                                    27

25 (2) INFORMATION FOR SEQ ID NO: 6:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 19 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
30           (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid
             (A) DESCRIPTION:   /desc = "LWN5039"


        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

     GCACATCATC ATCATAAGC                                             19
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(2) INFORMATION FOR SEQ ID NO: 7:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 23 base pairs
            (B) TYPE: nucleic acid
 5          (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid
            (A) DESCRIPTION:   /desc = "LWN5136"


        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

10  CCGGCGGATC CAAGGGGTGA TCG                                        23

    (2) INFORMATION FOR SEQ ID NO: 8:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 45 base pairs
            (B) TYPE: nucleic acid
15          (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid
            (A) DESCRIPTION:   /desc = "LWN2043"


        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

20  GGGGTACTAG TAACCCGGGC CCGGCGTAGA GGATCCATAC ACAAA               45

    (2) INFORMATION FOR SEQ ID NO: 9:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 36 base pairs
            (B) TYPE: nucleic acid
25          (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid
            (A) DESCRIPTION:   /desc = "LWN5232"


        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

30  GTCGGAGCTC ATTATTAATC TGTTCAGCAA TCGGGC                         36

    (2) INFORMATION FOR SEQ ID NO: 10:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 35 base pairs
            (B) TYPE: nucleic acid
35          (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear
```

(ii) MOLECULE TYPE: other nucleic acid
    (A) DESCRIPTION: /desc = "LWN5233"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GTCGGAGCTC TGCCTTTTAG TCCAGCTGAT TTCAC   35

5 (2) INFORMATION FOR SEQ ID NO: 11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 44 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
10   (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid
        (A) DESCRIPTION: /desc = "LWN7839"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GACGGGATCC CTGCAGTATC CAATTTATTT TTTTCTTAAC AAGG   44

15 (2) INFORMATION FOR SEQ ID NO: 12:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 40 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
20   (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid
        (A) DESCRIPTION: /desc = "LWN7840"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GACGGATTCA AAGCTTAAAG CACTTGCATA GGCTAATGCC   40

25 (2) INFORMATION FOR SEQ ID NO: 13:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 46 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
30   (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid
        (A) DESCRIPTION: /desc = "LWN7794"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GACGGGTACC ACGCGTTAAT CAATAAAAAA ACGCTGTGCG GTTAAA   46

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(2) INFORMATION FOR SEQ ID NO: 14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 43 base pairs
        (B) TYPE: nucleic acid
5   (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid
        (A) DESCRIPTION: /desc = "LWN7790"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

10 TATATATTTT AAAAATATCC CACGGTTCTT CAAATATTTC TCC   43

(2) INFORMATION FOR SEQ ID NO: 15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 44 base pairs
        (B) TYPE: nucleic acid
15   (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid
        (A) DESCRIPTION: /desc = "LWN7789"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

20 GGAGAAATAT TTGAAGAACC GTGGGATATT TTTAAAATAT ATAT   44

(2) INFORMATION FOR SEQ ID NO: 16:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 45 base pairs
        (B) TYPE: nucleic acid
25   (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid
        (A) DESCRIPTION: /desc = "LWN7788"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

30 CAAGTGTTCG CTTCGCTCTC ACGGAGCTGT AATATAAAAA CCTTC   45

(2) INFORMATION FOR SEQ ID NO: 17:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 45 base pairs
        (B) TYPE: nucleic acid
35   (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

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        (ii) MOLECULE TYPE: other nucleic acid
             (A) DESCRIPTION: /desc = "LWN7787"

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GAAGGTTTTT ATATTACAGC TCCGTGAGAG CGAAGCGAAC ACTTG   45

5 (2) INFORMATION FOR SEQ ID NO: 18:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 45 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
10   (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid
             (A) DESCRIPTION: /desc = "LWN7784"

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

CATATGATCA AATGGTTCGG ATCTGATTTT CCTCCTCTAA TATGC   45

15 (2) INFORMATION FOR SEQ ID NO: 19:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 55 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
20   (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid
             (A) DESCRIPTION: /desc = "LWN8197"

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GCATATTAGA GGAGGAAAAT CAGATCCGAA CCATTTGATC ATATGACAAG ATGTG   55

25 (2) INFORMATION FOR SEQ ID NO: 20:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 35 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
30   (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid
             (A) DESCRIPTION: /desc = "LWN7791"

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

GACGGAATTC CCGCGGTAAA TAGCAATAAA TTGGC   35

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(2) INFORMATION FOR SEQ ID NO: 21:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 base pairs
        (B) TYPE: nucleic acid
5   (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid
        (A) DESCRIPTION: /desc = "LWN7780"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

10 GACGGGTACC ACGCGTTAAT C   21

(2) INFORMATION FOR SEQ ID NO: 22:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
15   (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid
        (A) DESCRIPTION: /desc = "LWN5067"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

20 CCAGAACCTG TCAATCCACG   20

(2) INFORMATION FOR SEQ ID NO: 23:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 50 base pairs
        (B) TYPE: nucleic acid
25   (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid
        (A) DESCRIPTION: /desc = "LWN8524"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

30 GACTGAATTC GGATCCACGC GTATAATAAA GAATAATTAT TAATCTGTAG   50

(2) INFORMATION FOR SEQ ID NO: 24:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 42 base pairs
        (B) TYPE: nucleic acid
35   (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

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        (ii) MOLECULE TYPE: other nucleic acid
             (A) DESCRIPTION:   /desc = "LWN8528"


        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

    GACTAAGCTT GAGCTCCACT AATATTAATA AACTATCGAA GG                        42

  5 (2) INFORMATION FOR SEQ ID NO: 25:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 42 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
 10          (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid
             (A) DESCRIPTION:   /desc = "LWN8529"


        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

    GACTGAATTC CTGCAGGAGC TCAGTGAGAG CGAAGCGAAC AC                        42

 15 (2) INFORMATION FOR SEQ ID NO: 26:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 88 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
 20          (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid
             (A) DESCRIPTION:   /desc = "LWN8531"


        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

    GACTAAGCTT TGATCAAATG GTTGCGGCCG CGTCGACTCT AGACCCGGGT ACCAGATCTG     60

 25 GATCCTCGGG TTCTTCAAAT ATTTCTCC                                        88

    (2) INFORMATION FOR SEQ ID NO: 27:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 39 base pairs
             (B) TYPE: nucleic acid
 30          (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid
             (A) DESCRIPTION:   /desc = "LWN8518"
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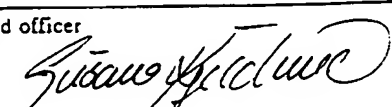
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

GACTAAGCTT ACGCGTTCGG GTTCTTCAAA TATTTCTCC   39

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "LWN8527"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

GACTGAATTC TGATCAAATG GTTCAGTGAG AGCGAAGCGA ACAC   44

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "LWN7191"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

GTTTTCCCAG TCACGAC   17

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "LWN8516"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

GACTGAATTC GGATCCACGC GTGAGTAGTT CAACAAACGG GCC   43

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 41 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: other nucleic acid
    (A) DESCRIPTION: /desc = "LWN8517"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

GACTAAGCTT GAGCTCCAAC ATGATTAACA ATTATTAGAG G   41

10 (2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 56 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
15   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
    (A) DESCRIPTION: /desc = "LWN8760"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

GACGGAATTC TCTAGAGTCG ACAGATCCGA ACCATTTGAT CATATGACAA GATGTG   56

20 (2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 40 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
25   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
    (A) DESCRIPTION: /desc = "LWN8761"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

GACGGAATTC GCGGCCGCGG TAAATAGCAA TAAATTGGCC   40

30 (2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 24 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
35   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
    (A) DESCRIPTION: /desc = "LWN4123"

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

AGCGGATAAC AATTTCACAC AGGA   24

(2) INFORMATION FOR SEQ ID NO: 35:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 34 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid
        (A) DESCRIPTION: /desc = "Tnase1"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

TGACGGATCC ACGCGTGGCG CACTCCCGTT CTGG   34

(2) INFORMATION FOR SEQ ID NO: 36:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 36 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid
        (A) DESCRIPTION: /desc = "Tnase2"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

GTACGGATCC ACGCGTAAAG GCACCTTTGG TCACGG   36
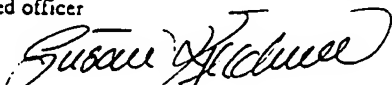
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page **40** , lines **25-27**	

B. IDENTIFICATION OF DEPOSIT   Further deposits are identified on an additional sheet ☒
Name of depositary institution   DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELL-KULTUREN GmbH
Address of depositary institution *(including postal code and country)*   Mascheroder Weg 1b, D-38124 Braunschweig, Federal Republic of Germany

Date of deposit
    22 December 1995
	Accession Number
	    DSM10445

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)*   This information is continued on an additional sheet ☐
In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*
The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description

on page   **47**   , line s   **10-12**   .

B. IDENTIFICATION OF DEPOSIT   Further deposits are identified on an additional sheet [X]

Name of depositary institution

    DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELL-KULTUREN GmbH

Address of depositary institution *(including postal code and country)*

    Mascheroder Weg 1b, D-38124 Braunschweig, Federal Republic of Germany

Date of deposit	Accession Number
22 December 1995	DSM10444

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)*   This information is continued on an additional sheet ☐

    In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description
on page ___47___ , line s _25-27_ .

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution

DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELL-KULTUREN GmbH

Address of depositary institution *(including postal code and country)*

Mascheroder Weg 1b, D-38124 Braunschweig, Federal Republic of Germany

Date of deposit	Accession Number
22 December 1995	DSM10446

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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CLAIMS

1. A DNA construct comprising the structure IR(1)-P-R-M2-R-
IR(2) or IR(1)-R-M2-R-P-IR(2), wherein
IR(1) and IR(2) denote transposase target sequences,
5 P a DNA sequence of interest,
R a target sequence for a site-specific recombination enzyme,
and
M2 a selectable marker gene,
said structure being associated with a transposase gene T which
10 is located on either side of and outside said structure.

2. A DNA construct comprising the structure IR(1)-R-M2-T-R-P-
IR(2), IR(1)-P-R-M2-T-R-IR(2), IR(1)-R-T-M2-R-P-IR(2) or IR(1)-
P-R-T-M2-R-IR(2), wherein

IR(1) and IR(2) denote transposase target sequences,
15 P a DNA sequence of interest,
R a target sequence for a site-specific recombination enzyme,
M2 a selectable marker gene, and
T a transposase gene T.

3. The DNA construct according to claim 1 or 2, wherein R is a
20 res-site from plasmid pAMβ1 or a lox site from phage P1.

4. A DNA construct comprising the structure IR(1)-P-R'-M2-R''-
IR(2) or IR(1)-R'-M2-R''-P-IR(2), wherein
IR(1) and IR(2) denote transposase target sequences,
P a DNA sequence of interest,
25 R' and R'' parallel repeat sequences, and
M2 a selectable marker gene,
said structure being associated with a transposase gene T which
is located on either side of and outside said structure.

5. A DNA construct comprising the structure IR(1)-R'-M2-T-R''-
30 P-IR(2), IR(1)-P-R'-M2-T-R''-IR(2), IR(1)-R'-T-M2-R''-P-IR(2)
or IR(1)-P-R'-T-M2-R''-IR(2), wherein

IR(1) and IR(2) denote transposase target sequences,

P a DNA sequence of interest,

R' and R'' parallel repeat sequences,

M2 a selectable marker gene, and

5 T a transposase gene T.

6. The DNA construct according to any of claims 1-5, which further comprises a selectable marker gene, M1, which may be different from or identical with M2, and which is located on either side of and outside the structure located within and 10 comprising IR(1) and IR(2).

7. The DNA construct according to any of the preceeding claims, which is capable of being transposed in a gram-positive bacterium, in particular a cell of <u>Bacillus</u>.

8. The DNA construct according to any of claims 1-7, which 15 further comprises a cis-acting DNA sequence required for the transfer of the DNA construct into a recipient cell by con-jugation in the presence of a trans-acting mobilizing element.

9. The DNA construct according to any of claims 1-8, in which the DNA sequence P encodes a polypeptide of interest.

20 10. The DNA construct according to claim 9, in which the DNA sequence P encodes a translocated polypeptide.

11. The DNA construct according to claim 10, wherein the DNA sequence P encodes a secreted polypeptide.

12. The DNA construct according to claim 9, wherein the 25 polypeptide is an enzyme.

13. The DNA construct according to claim 12, wherein the enzyme is selected from the group consisting of an amylolytic enzyme, a lipolytic enzyme, a proteolytic enzyme, a cellulytic enzyme, an oxidoreductase or a plant cell-wall degrading enzyme.

14. The DNA construct according to claim 10, wherein the polypeptide is PrsA.

15. A vector comprising the DNA construct according to any of claims 1-14.

16. The vector according to claim 15, which comprises a conditional origin of replication.

17. The vector according to claim 16, in which the conditional origin of replication is a temperature sensitive origin of replication.

18. A bacterial cell, which in its constitutive DNA has integrated at least two copies of a DNA construct comprising the following structure IR(1)-P-IR(2), in which
IR(1) and IR(2) denote transposase target sequences, and
P a DNA sequence of interest, the structure further comprising a DNA sequence R being a target sequence for a site-specific recombination enzyme which is present as a result of excision of a DNA fragment between two R sequences by the action of a resolvase enzyme or the DNA sequence resulting from the homologous recombination between the DNA sequences R'/R''.

19. The cell according to claim 18 which is free from any undesired selectable marker gene.

20. The cell according to claim 19, which is free from a DNA sequence encoding a selectable antibiotic-resistance marker.

21. The cell according to any of claims 18-20, which is a cell of a gram-positive bacterium.

22. The cell according to claim 21, which is a cell of a Bacillus sp. or a Lactobacillus sp.

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23. The cell according to claim 22, which is a cell of Bacillus sp. selected from the group consisting of Bacillus subtilis, Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus circulans, Bacillus lautus, Bacillus megaterium, Bacillus thuringiensis.

24. The cell according to any of claims 18-23, wherein the DNA sequence P encodes a polypeptide of interest.

25. The cell according to claim 24, wherein the DNA sequence P encodes a translocated polypeptide.

26. The cell according to claim 20, wherein the DNA sequence P encodes a secreted polypeptide.

27. The cell according to claim 24, wherein the polypeptide is an enzyme.

28. The cell according to claim 27, wherein the enzyme is selected from the group consisting of an amylolytic enzyme, a lipolytic enzyme, a proteolytic enzyme, a cellulytic enzyme, an oxidoreductase or a plant cell-wall degrading enzyme.

29. A method of constructing a bacterial cell, which in its genomic DNA has integrated more than one copy of a DNA sequence of interest, and which is free from a DNA sequence encoding an undesired selectable marker, which method comprises

a) introducing a first vector comprising a DNA construct according to any of claims 1, 3 and 6-14 comprising the structure IR(1)-P-R-M2-R-IR(2) or IR(1)-R-M2-R-P-IR(2) in association with with a tranposase gene T and optionally a selectable marker gene M1 into the host cell,

b) selecting for cells being M1⁻, M2⁺, which in their genome
comprises the structure IR(1)-P-R-M2-R-IR(2) or IR(1)-R-M2-R-P-
IR(2),

c) introducing a second vector comprising a DNA sequence
5 encoding a site specific recombinase into the cells selected in
step b) so as to excise the structure R-M2 or M2-R from the
genome of the cell whereby cells are obtained having integrated
the structure IR(1)-R-P-IR(2) or IR(1)-P-R-IR(2),

d) curing the cells resulting from step c) for the second
10 plasmid, and optionally

e) repeating steps a-d one or more times to produce bacterial
cells comprising one or more additional copies of the structure
IR(1)-R-P-IR(2) or IR(1)-P-R-IR(2).

30. A method of constructing a bacterial cell, which in its
15 genomic DNA has integrated more than one copy of a DNA sequence
of interest, and which is free from a DNA sequence encoding an
undesired selectable marker, which method comprises

a) introducing a first vector comprising a DNA construct
according to any of claims 2, 3 and 6-14 comprising the struc-
20 ture IR(1)-R-M2-T-R-P-IR(2), IR(1)-P-R-M2-T-R-IR(2), IR(1)-R-T-
M2-R-P-IR(2) or IR(1)-P-R-T-M2-R-IR(2) optionally in associa-
tion with a selectable marker gene M1 into the host cell,

b) selecting for cells being M1⁻, M2⁺, which in their genome
comprises one of the structures identified in a),

25 c) selecting for cells having an increased number of copies of
the marker gene M2,

d) introducing a second vector comprising a DNA sequence
encoding a site specific recombinase into the cells selected in
step b) so as to excise the structure R-M2-T, R-T-M2, M2-T-R or

T-R-M2 from the genome of the cell whereby cells are obtained having integrated the structure IR(1)-R-P-IR(2) or IR(1)-P-R-IR(2),

e) curing the cells resulting from step d) for the second
5 plasmid, and optionally

f) repeating steps a-e one or more times to produce bacterial cells comprising one or more additional copies of the structure IR(1)-R-P-IR(2) or IR(1)-P-R-IR(2).

31. The method according to claim 29 or 30, wherein the second
10 plasmid is curable.

32. A method for constructing a bacterial cell, which in its genomic DNA has integrated more than one copy of a DNA sequence of interest, and which is free from a DNA sequence encoding an undesired selectable marker, which method comprises

15 a) introducing a first vector comprising a DNA construct according to any of claims 4 and 6-14 comprising the structure IR(1)-P-R'-M2-R''-IR(2) or IR(1)-R'-M2-R''-P-IR(2) in association with a tranposase gene T and optionally a selectable marker gene M1 into the host cell, in which R' and R'' denote parallel
20 repeat sequences,

b) selecting for cells being M1⁻, M2⁺, which in their genome comprises the structure IR(1)-P-R'-M2-R''-IR(2) or IR(1)-R'-M2-R''-P-IR(2),

c) allowing homologous recombination between the DNA sequences
25 R' and R'' to take place so as to excise the selectable marker gene M2, whereby cells are obtained having integrated the structure IR(1)-R'/R''-P-IR(2) or IR(1)-P-R'/R''-IR(2) (wherein R'/R'' denotes the common recombination sequence), and optionally

d) repeating steps a-c one or more times to produce bacterial
cells comprising one or more additional copies of the DNA
structure IR(1)-R'/R''-P-IR(2) or IR(1)-P-R'/R''-IR(2).

33. A method for constructing a bacterial cell, which in its
5 genomic DNA has integrated more than one copy of a DNA sequence
of interest, and which is free from a DNA sequence encoding an
undesired selectable marker, which method comprises

a) introducing a first vector comprising a DNA construct
according to any of claims 5-14 comprising the structure IR(1)-
10 R'-M2-T-R''-P-IR(2), IR(1)-P-R'-M2-T-R''-IR(2), IR(1)-R'-T-M2-
R''-P-IR(2) or IR(1)-P-R'-T-M2-R''-IR(2) optionally in associa-
tion with a selectable marker gene M1 into the host cell, in
which R' and R'' denote parallel repeat sequences,

b) selecting for cells being M1⁻, M2⁺, which in their genome
15 comprises the relevant structure identified in a),

c) selecting for cells having an increased number of copies of
the selectable marker gene M2,

d) allowing homologous recombination between the DNA sequences
R' and R'' to take place so as to excise the selectable marker
20 gene M2 and the transposase gene T, whereby cells are obtained
having integrated the structure IR(1)-R'/R''-P-IR(2) or IR(1)-
P-R'/R''-IR(2) (wherein R'/R'' denotes the common recombination
sequence), and optionally

e) repeating steps a-d one or more times to produce bacterial
25 cells comprising one or more additional copies of the DNA
structure IR(1)-R'/R''-P-IR(2) or IR(1)-P-R'/R''-IR(2).

34. The method according to claim 32 or 33, wherein the
selectable marker gene M2 is a counterselectable marker, such
as a amdS gene or a thy gene, and wherein, subsequent to step

c) selection is made for the absence of the counterselectable marker.

35. The method according to any of claims 29-34, wherein step a) is accomplished by conjugation, the first vector additional-
5 ly comprising a cis-acting DNA sequence required for the transfer of the vector into the bacterial cell by conjugation in the presence of a trans-acting mobilizing element, the first vector being present in a population of donor cells further comprising at least one DNA sequence encoding said trans-acting
10 mobilizing element, the popolation of donor cells being mixed with a population of recipient bacterial cells under conditions allowing the vector to be transferred from the population of the donor cells to the population of recipient cells by con-jugation.

15 36. The method according to any of claims 29-35, wherein the DNA construct introduced according to step a) further comprises an origin of replication.

37. The method according to claim 36, wherein the origin of replication is conditional.

20 38. The method according to claim any of claims 29-37, in which steps a)-e) is repeated one or more times.

39. The method according to any of claims 29-38, wherein the DNA sequence R and the DNA encoding the site-specific recombi-nase are derived from plasmid pAMβ1.

25 40. The method according to any of claims 29-39, wherein the integrated DNA construct IR(1)-P-IR(2), optionally further comprising R or R'/R'', is amplified so as to obtain cells comprising two or more copies of said DNA construct.

41. The method according to any of claims 29-40, wherein the
30 cell to be constructed is a cell of a gram-positive bacterium.

42. The method according to claim 41, wherein the cell is a cell of a <u>Bacillus</u> sp. or a <u>Lactobacillus</u> sp.

43. The method according to claim 42, wherein the cell is a cell of <u>Bacillus</u> sp. selected from the group consisting of <u>Bacillus subtilis</u>, <u>Bacillus licheniformis</u>, <u>Bacillus lentus</u>, <u>Bacillus brevis</u>, <u>Bacillus stearothermophilus</u>, <u>Bacillus alkalophilus</u>, <u>Bacillus amyloliquefaciens</u>, <u>Bacillus coagulans</u>, <u>Bacillus circulans</u>, <u>Bacillus lautus</u>, <u>Bacillus megaterium</u>, <u>Bacillus thuringiensis</u>.

44. A method of producing a protein of interest encoding by a DNA sequence P, which method comprises cultivating a cell according to any of claims 18-28 and/or produced by the method according to any of claims 29-43, which cell comprises more than one copy of the DNA sequence P, under conditions suitable for producing the polypeptide, and recovering the polypeptide from the resulting cell broth.

45. A marker-free cell of a gram-positive bacterium, in particular of a <u>Bacillus</u> sp., comprising muliple copies of a DNA sequence P of interest.
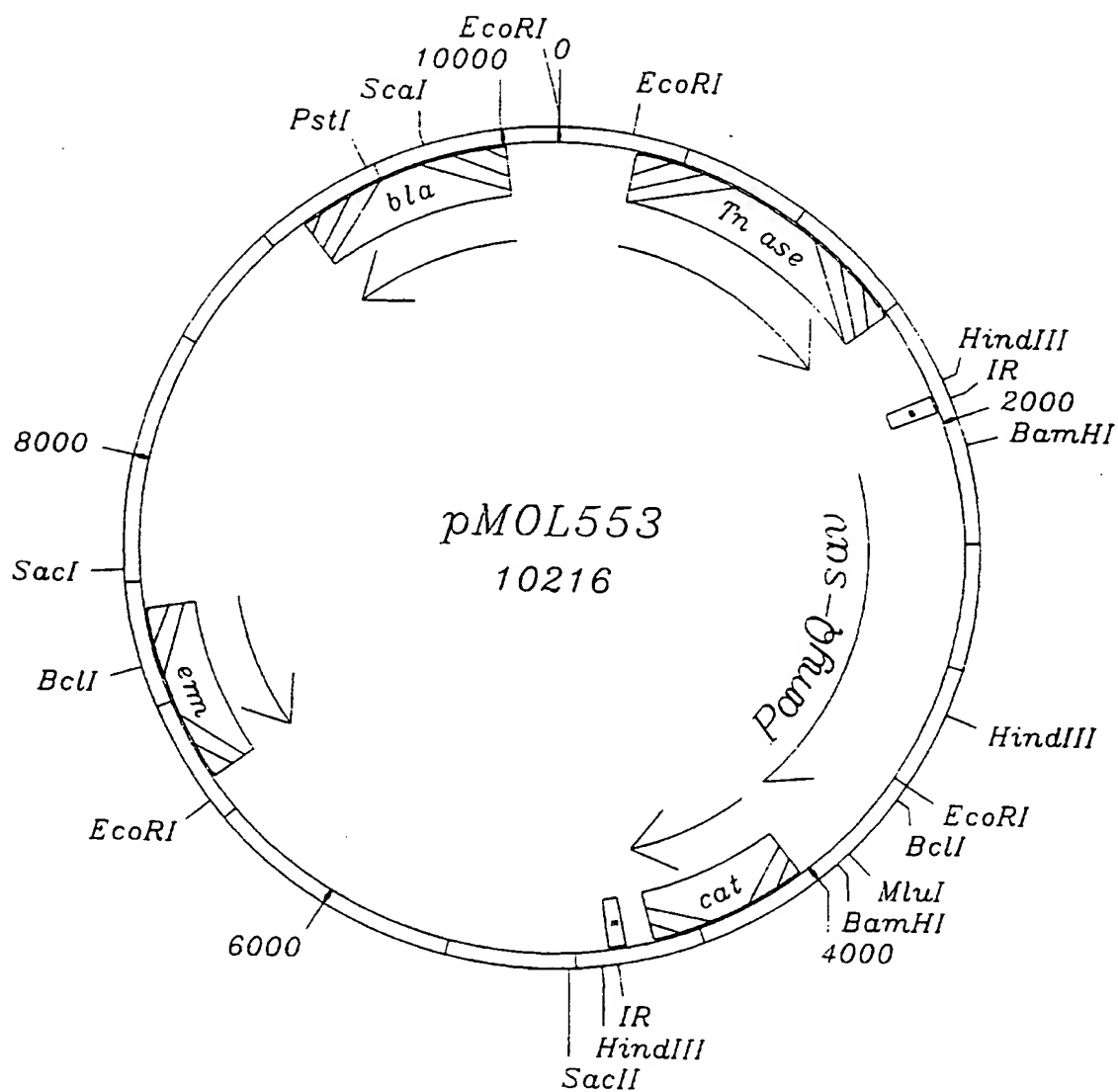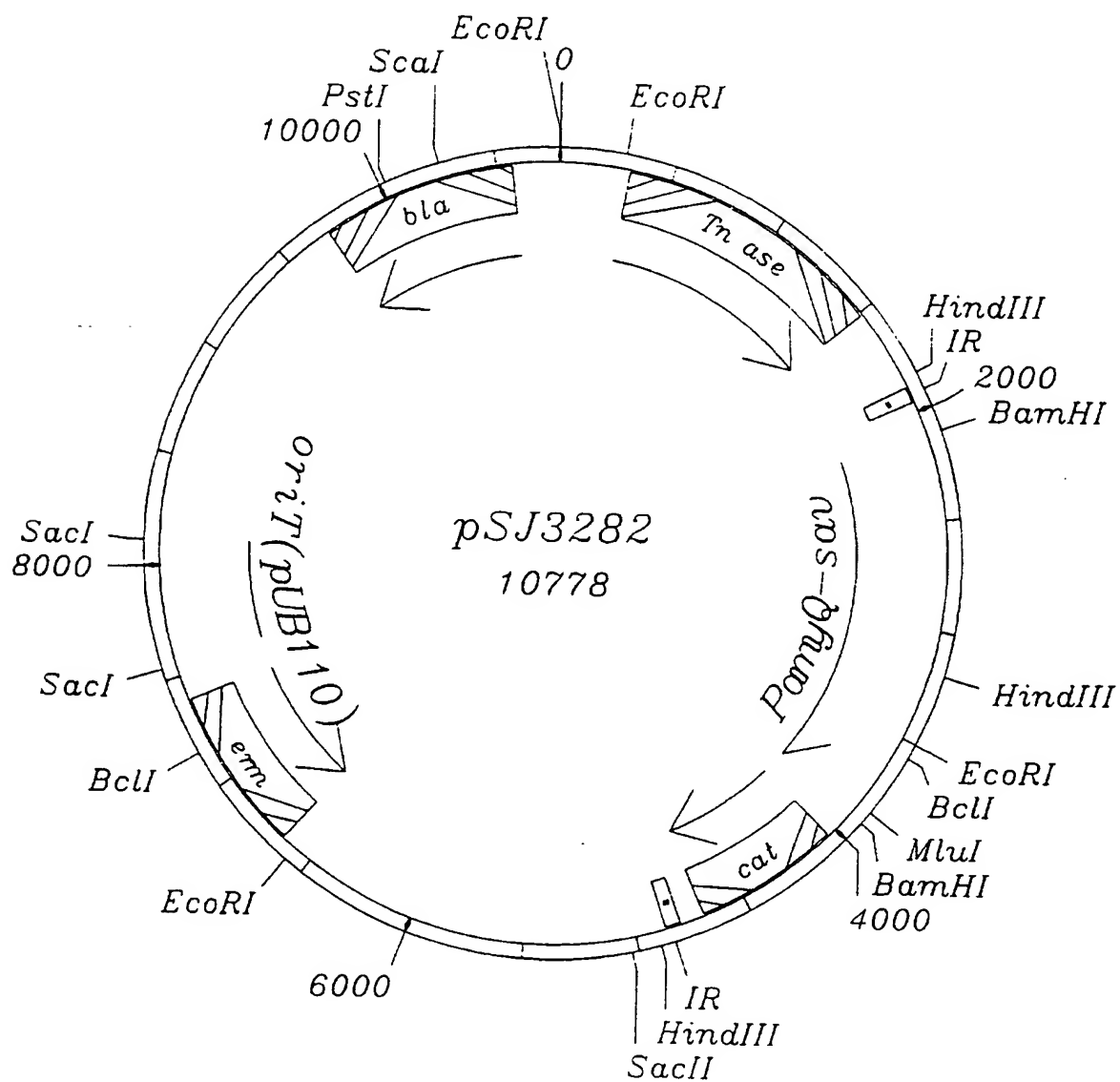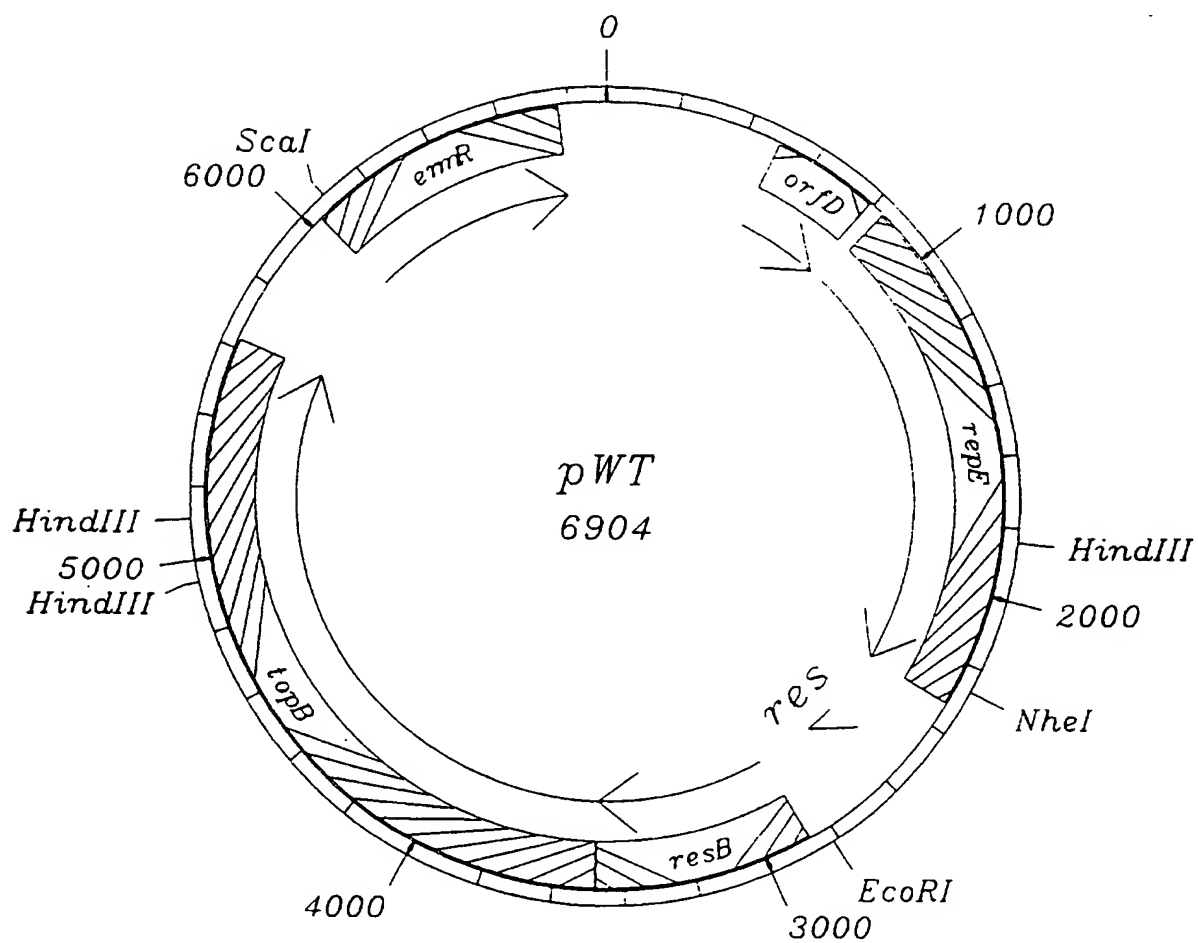
Fig. 1

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Fig. 2

Fig. 3

Fig. 4

Fig. 5

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Fig. 6

BglII 0

7000

kan

SphI
1000

SacI

oriT(pUB110)

PamyL

PstI

SacI
6000

repf

pSJ3279–pSJ3281
7763

resB

2000

SphI

HindIII
SphI
PstI

5000
+ ori pE194

erm

BclI
3000

PstI

SacI

4000

Fig. 7

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Fig. 8

Fig. 9

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Fig. 10

SJ3470   SJ3469   SJ3468   SJ3467   SJ3466   SJ3465
  ○   ○   ○   ○   ○   ○

                    DN1885   SJ3461   SJ3472   SJ3471
  ○   ○   ○   ○   ○   ○

  10   5   2   1   0   2.5   Savinase standard
  ○   ○   ○   ○   ○   ○   NPU/ml

                    SJ3540   SJ3539   SJ3538   SJ3537
  ○   ○   ○   ○   ○   ○

SJ3546   SJ3545   SJ3544   SJ3543   SJ3542   SJ3541
  ○   ○   ○   ○   ○   ○
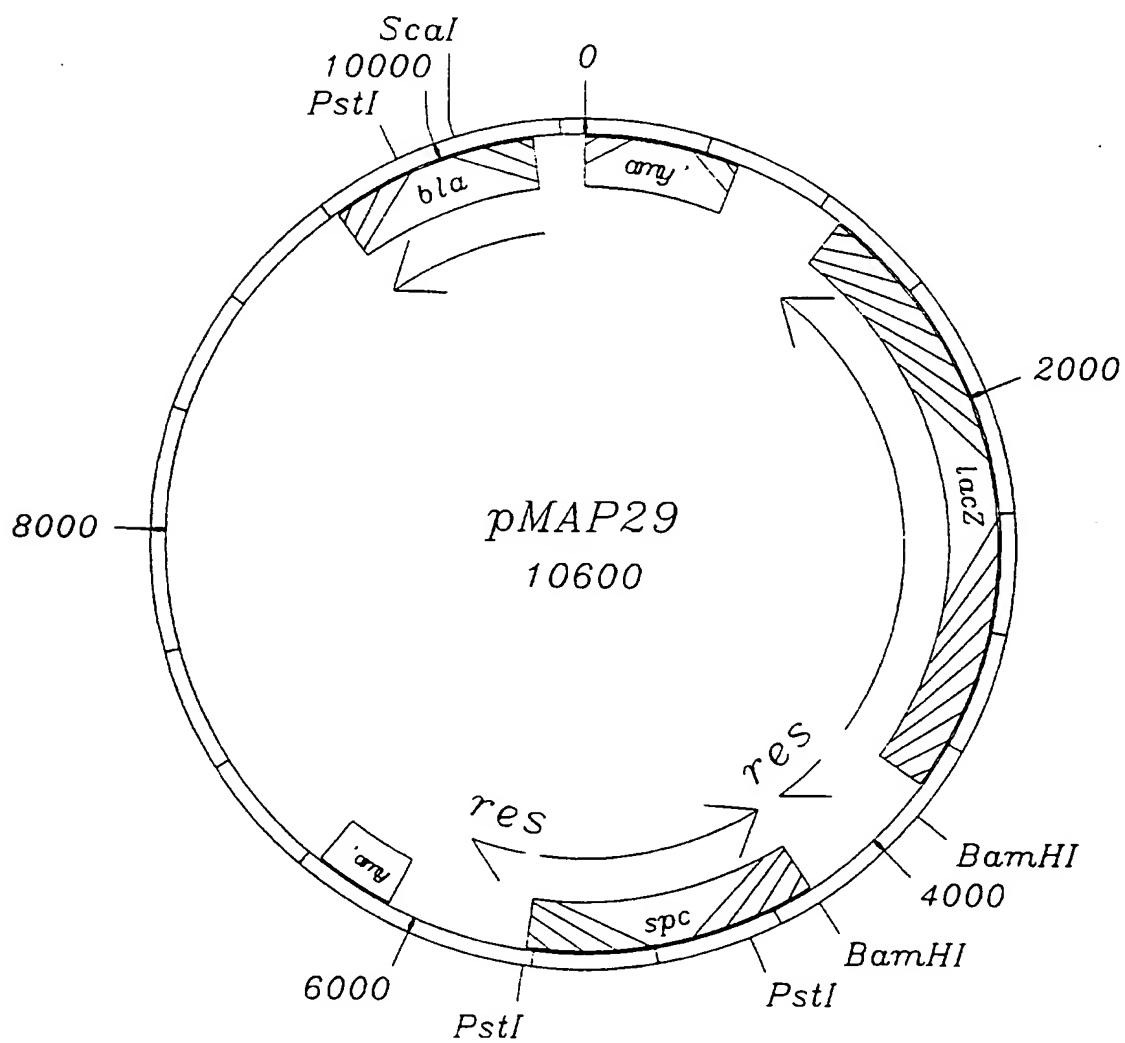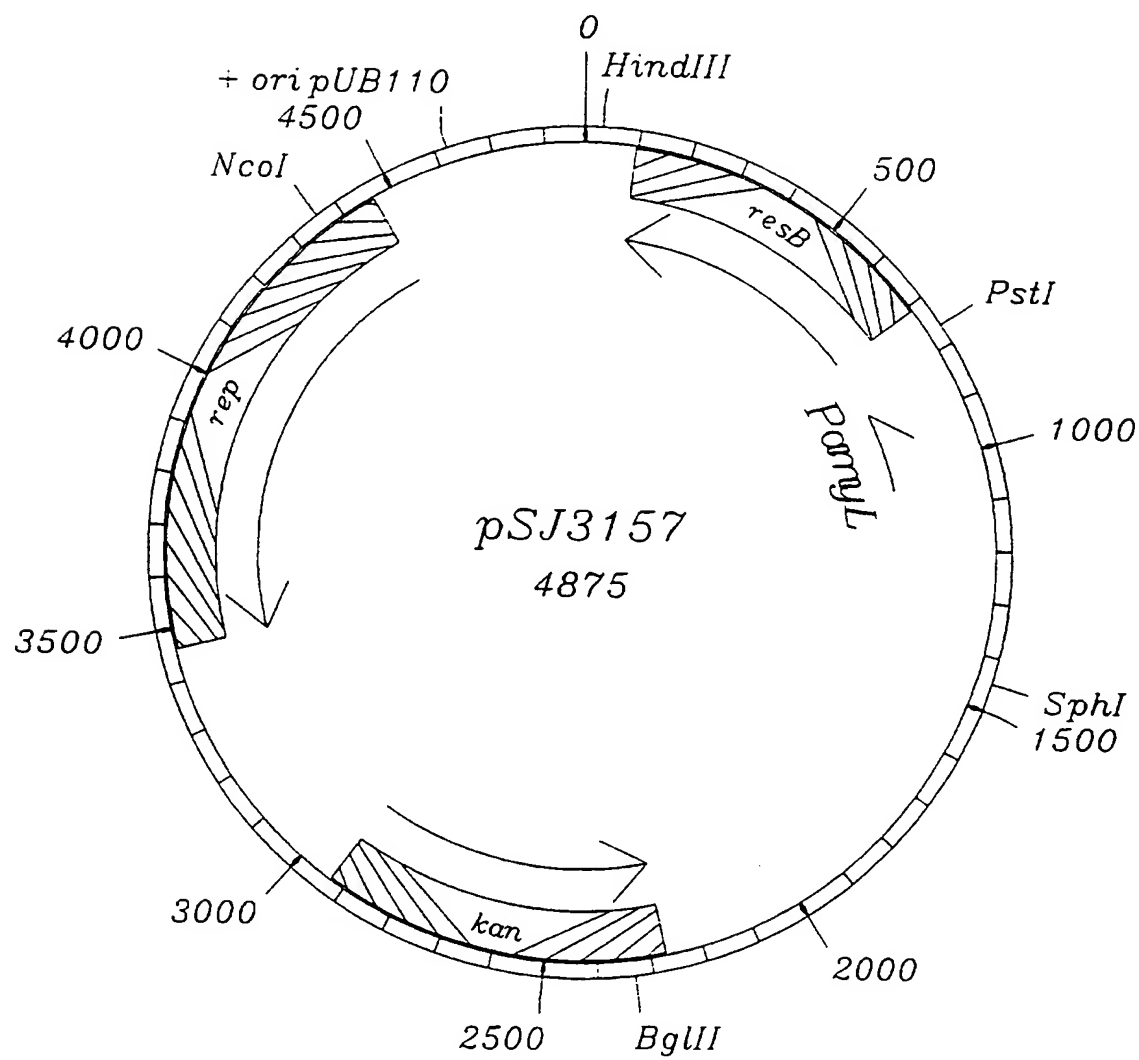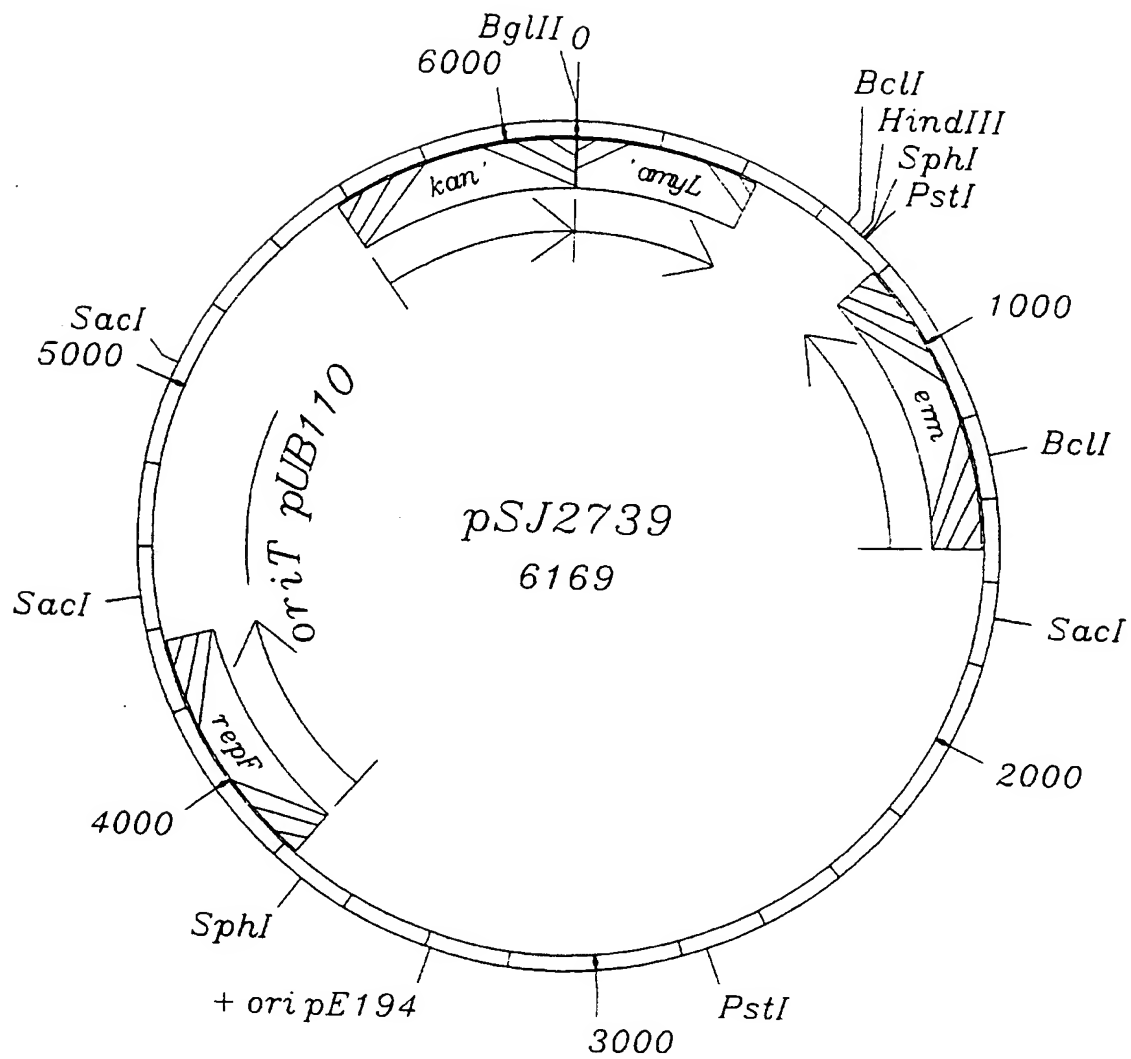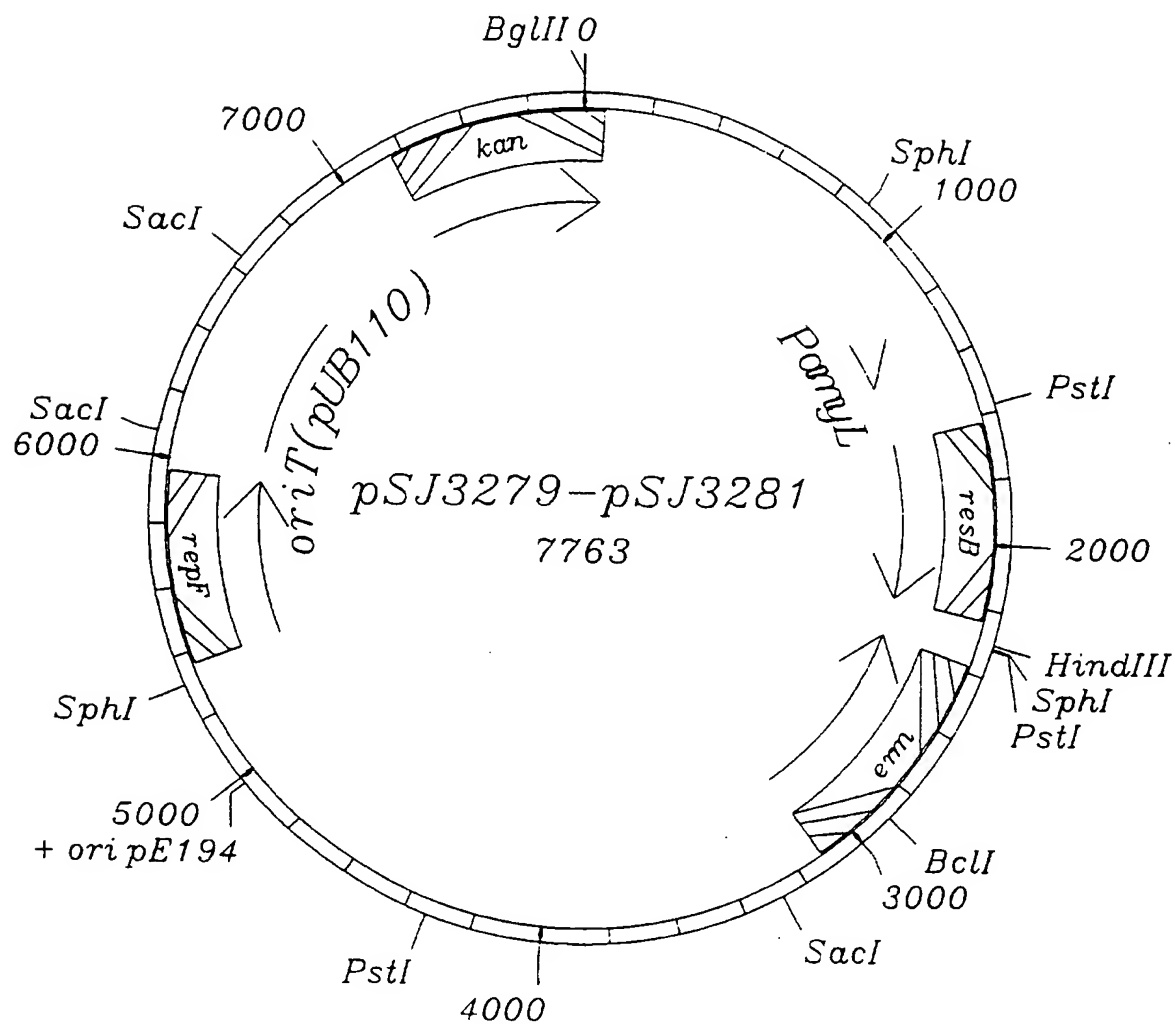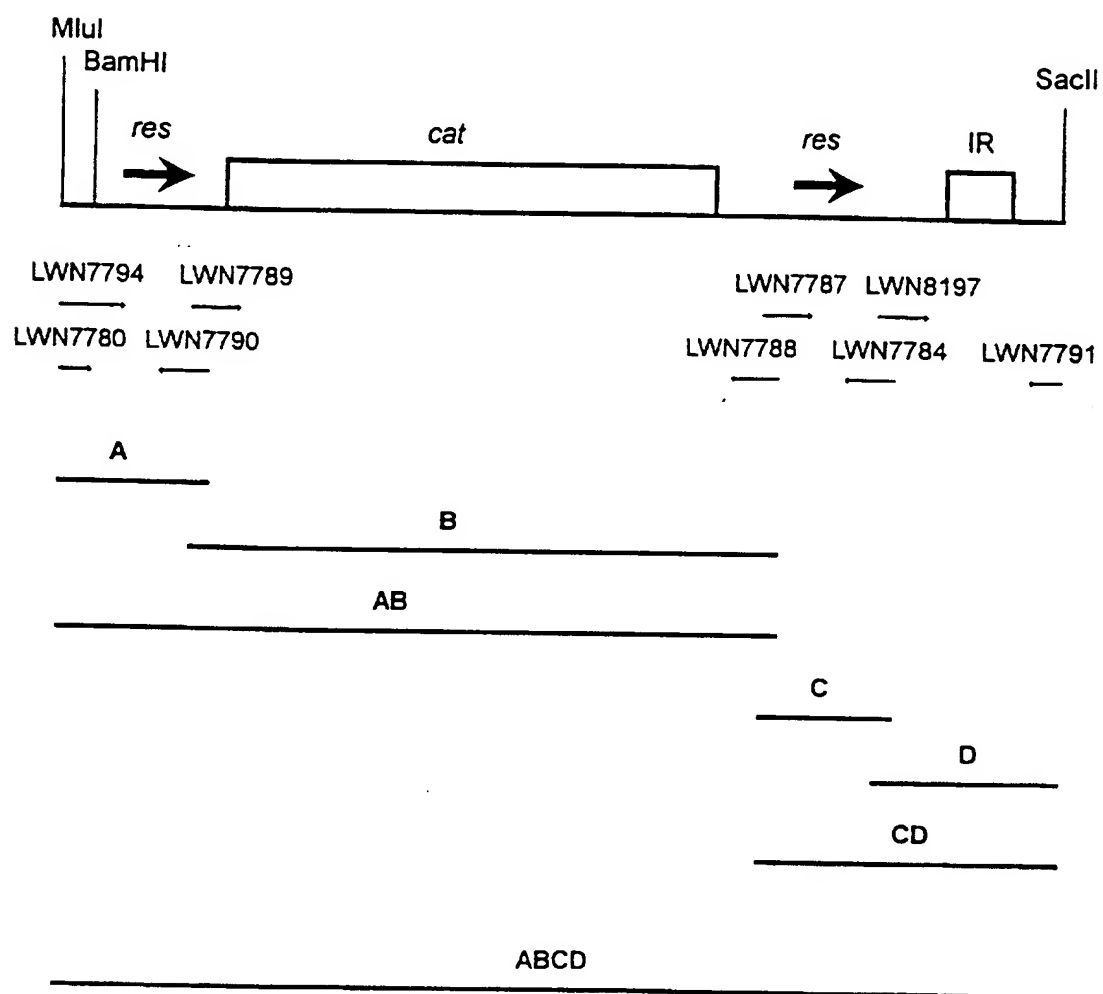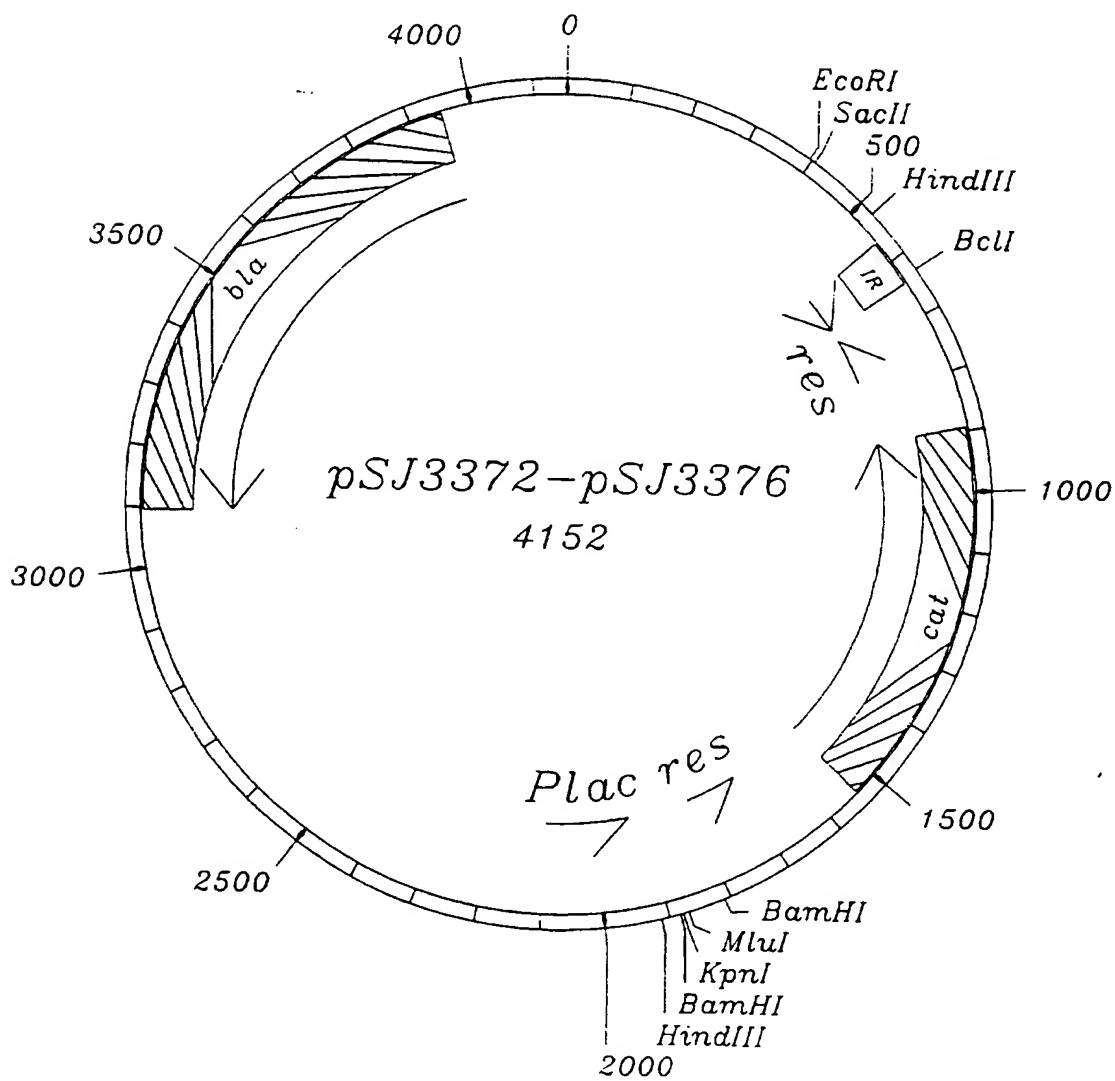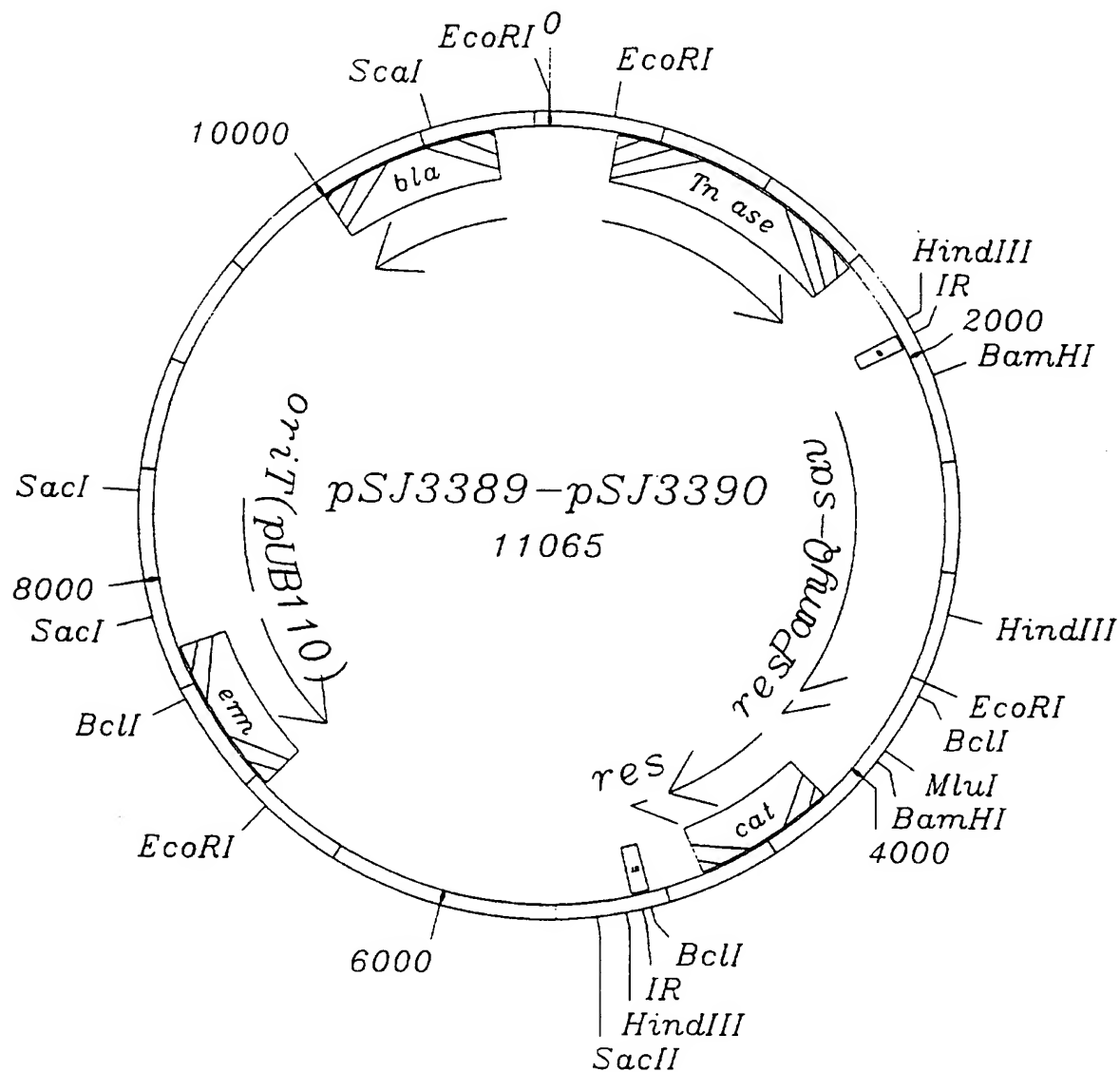
Fig. 11A

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2   4   6   8   10   Savinase standard
○   ○   ○   ○   ○   NPU/ml

SJ3465   SJ3537   SJ3539   SJ3541   SJ3546
○   ○   ○   ○   ○

SJ3461   SJ3565   SJ3566   SJ3567   SJ3568
○   ○   ○   ○   ○
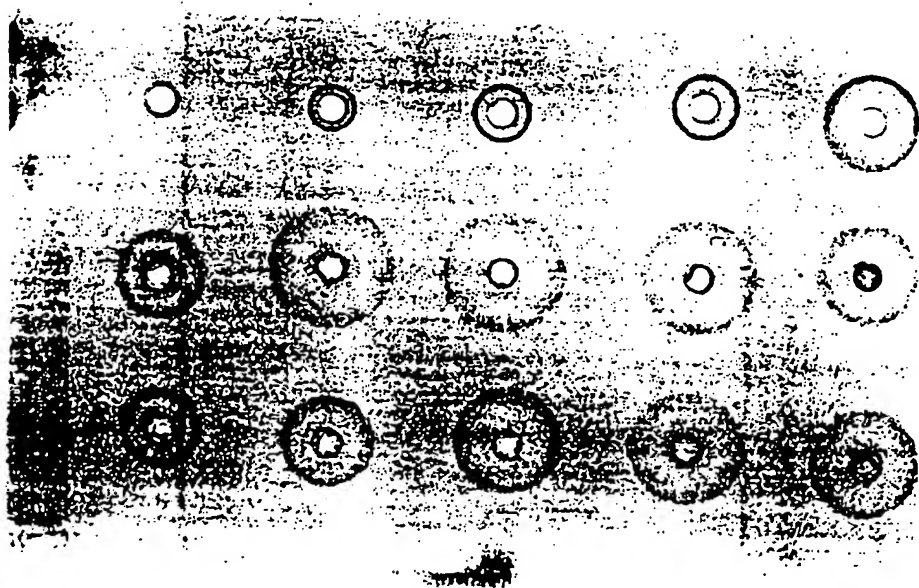
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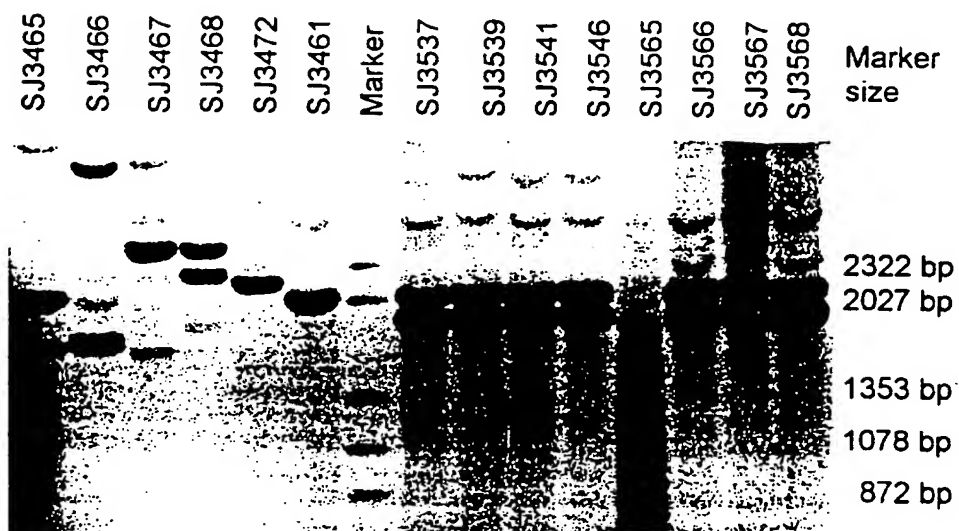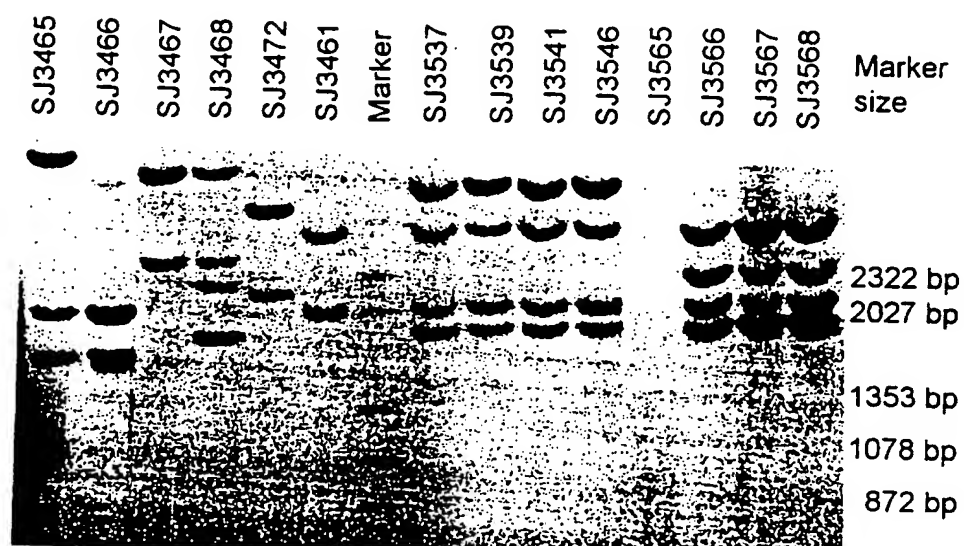
Fig. 12A

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Fig. 12B
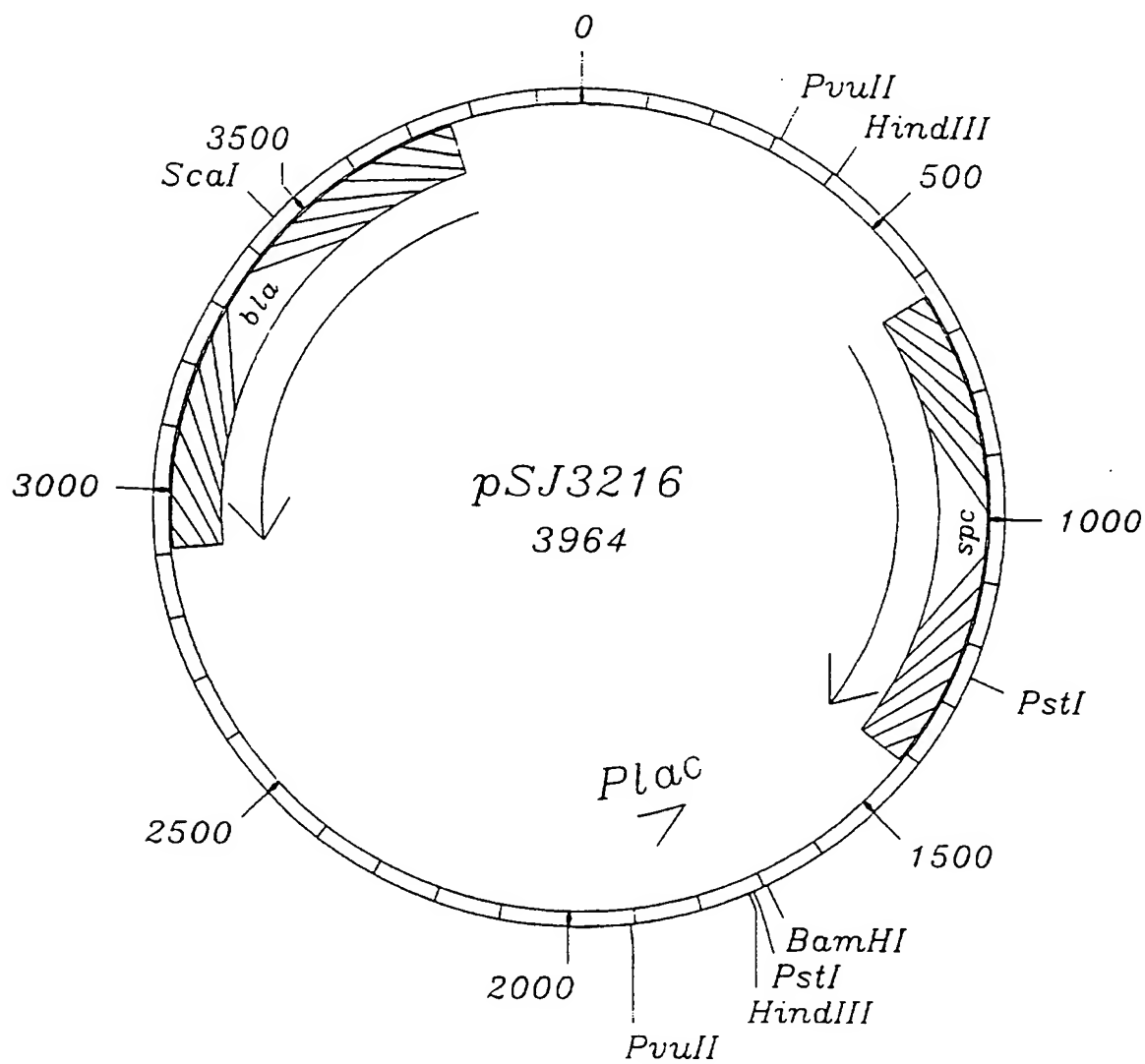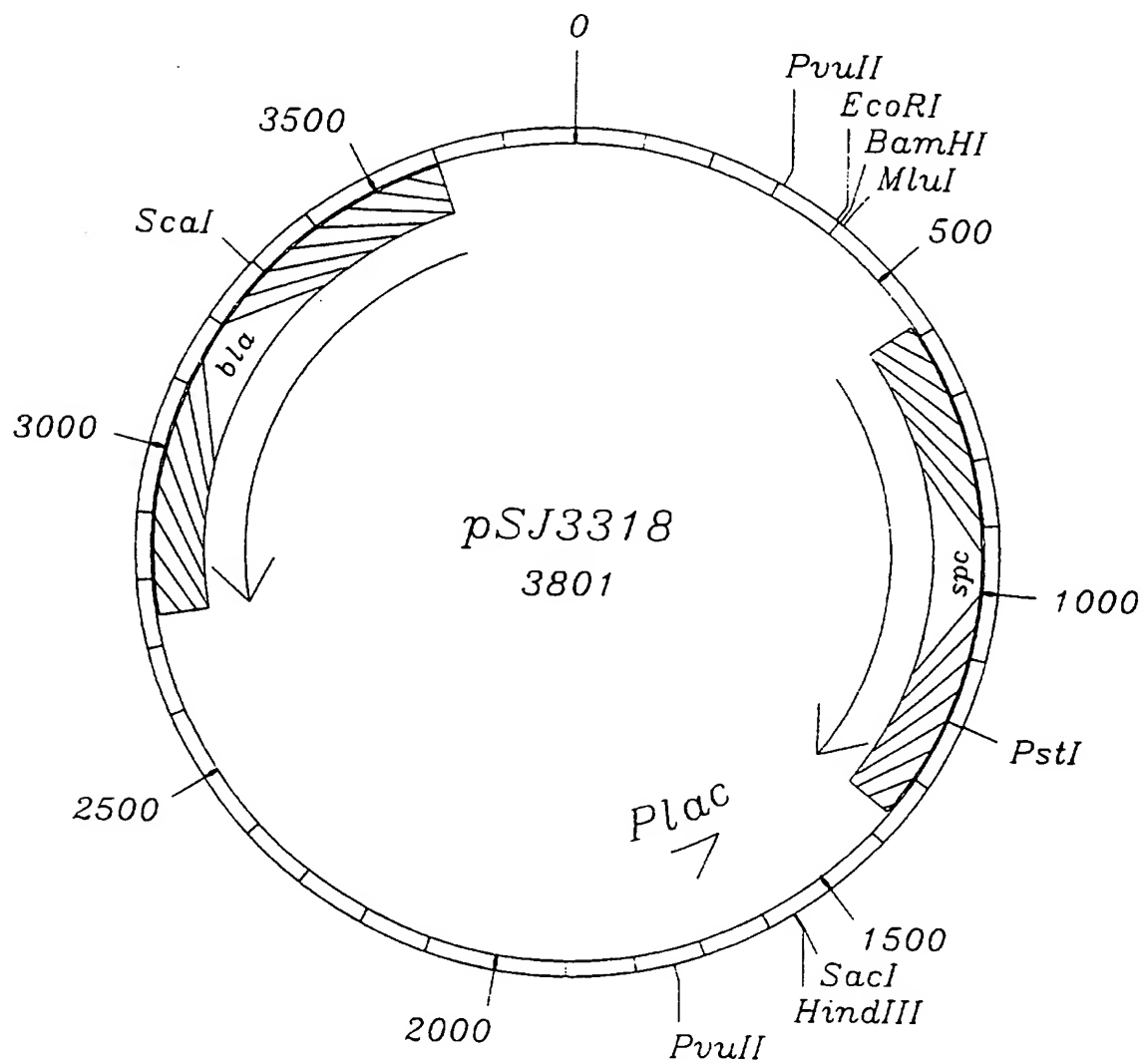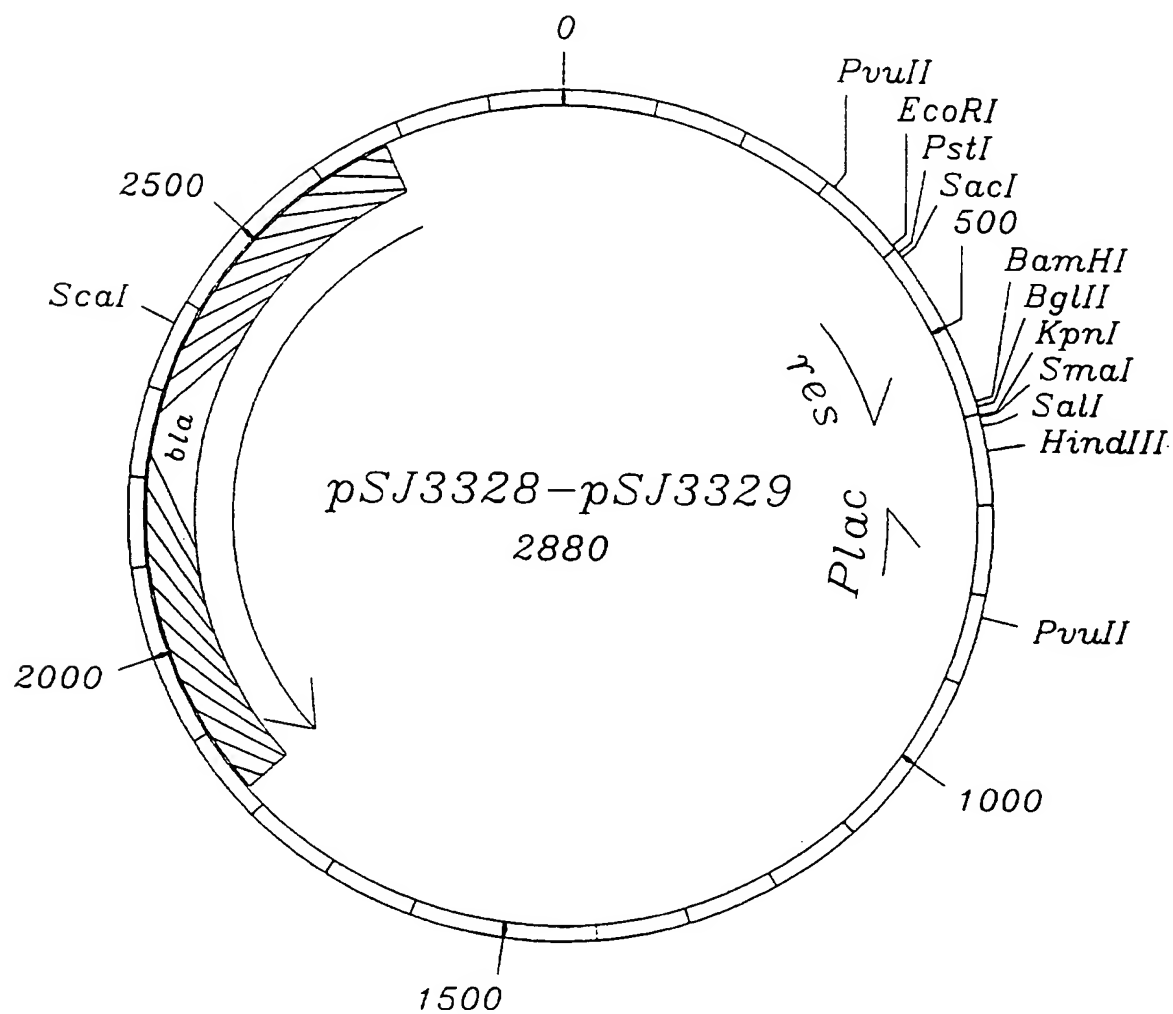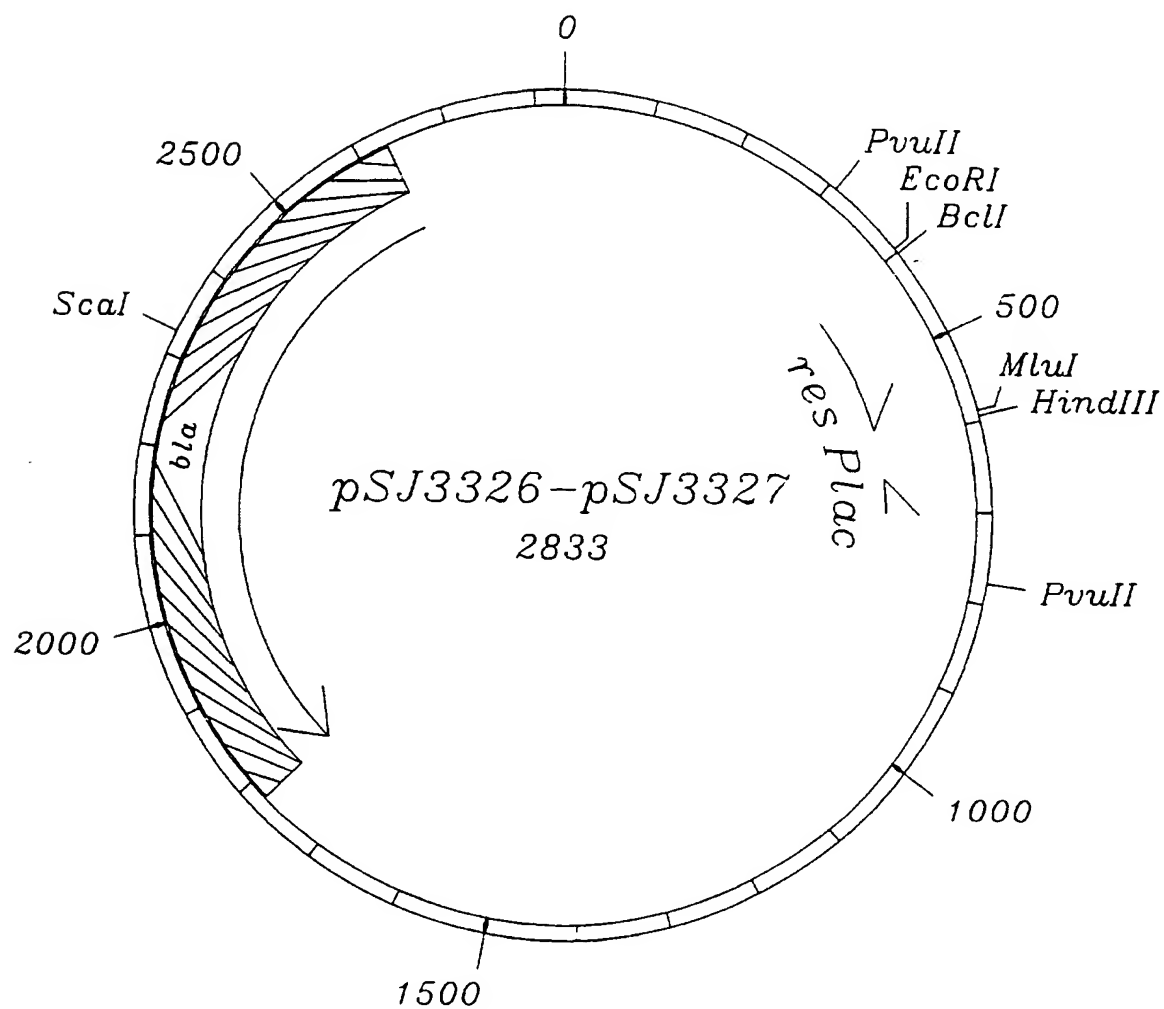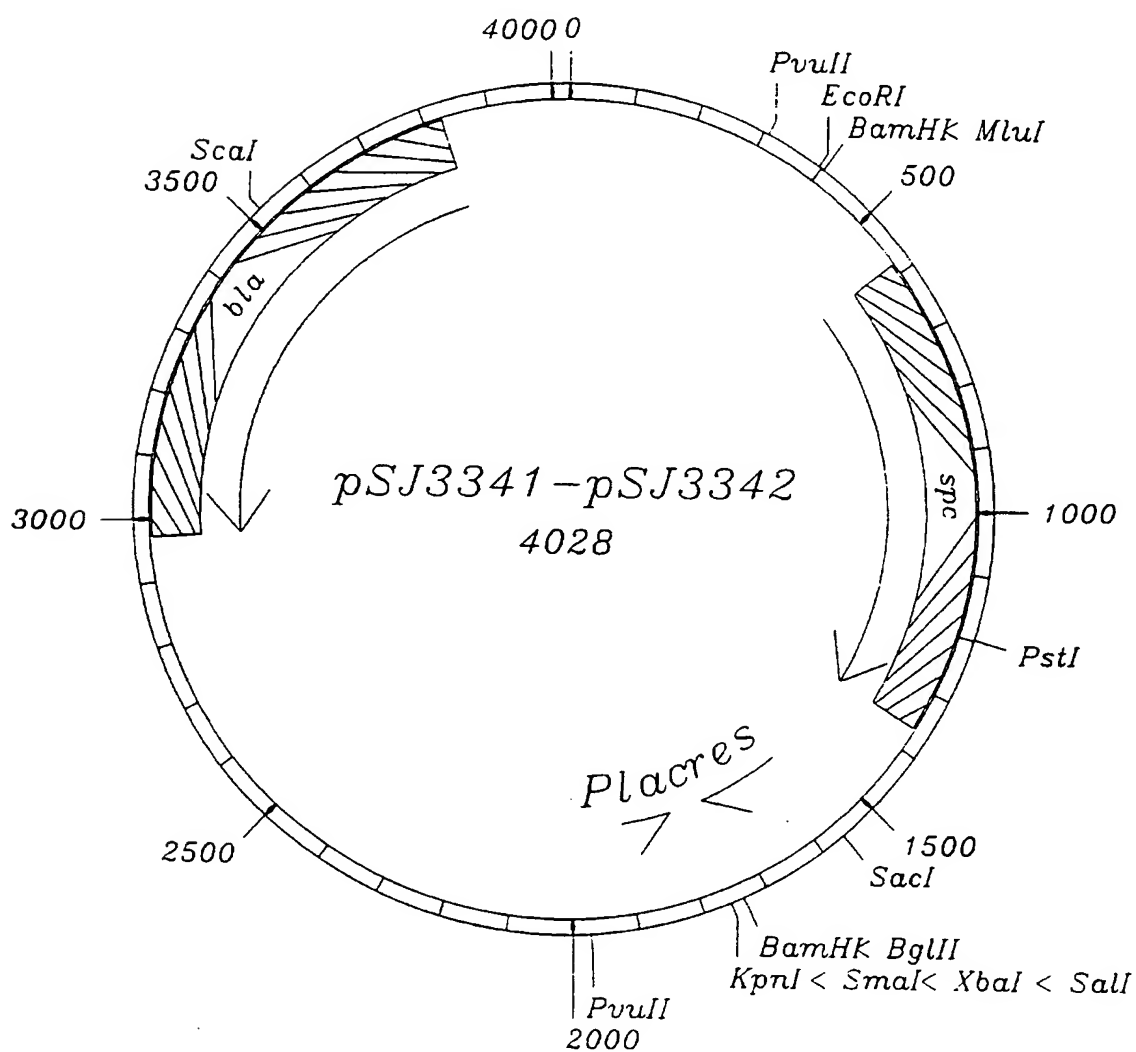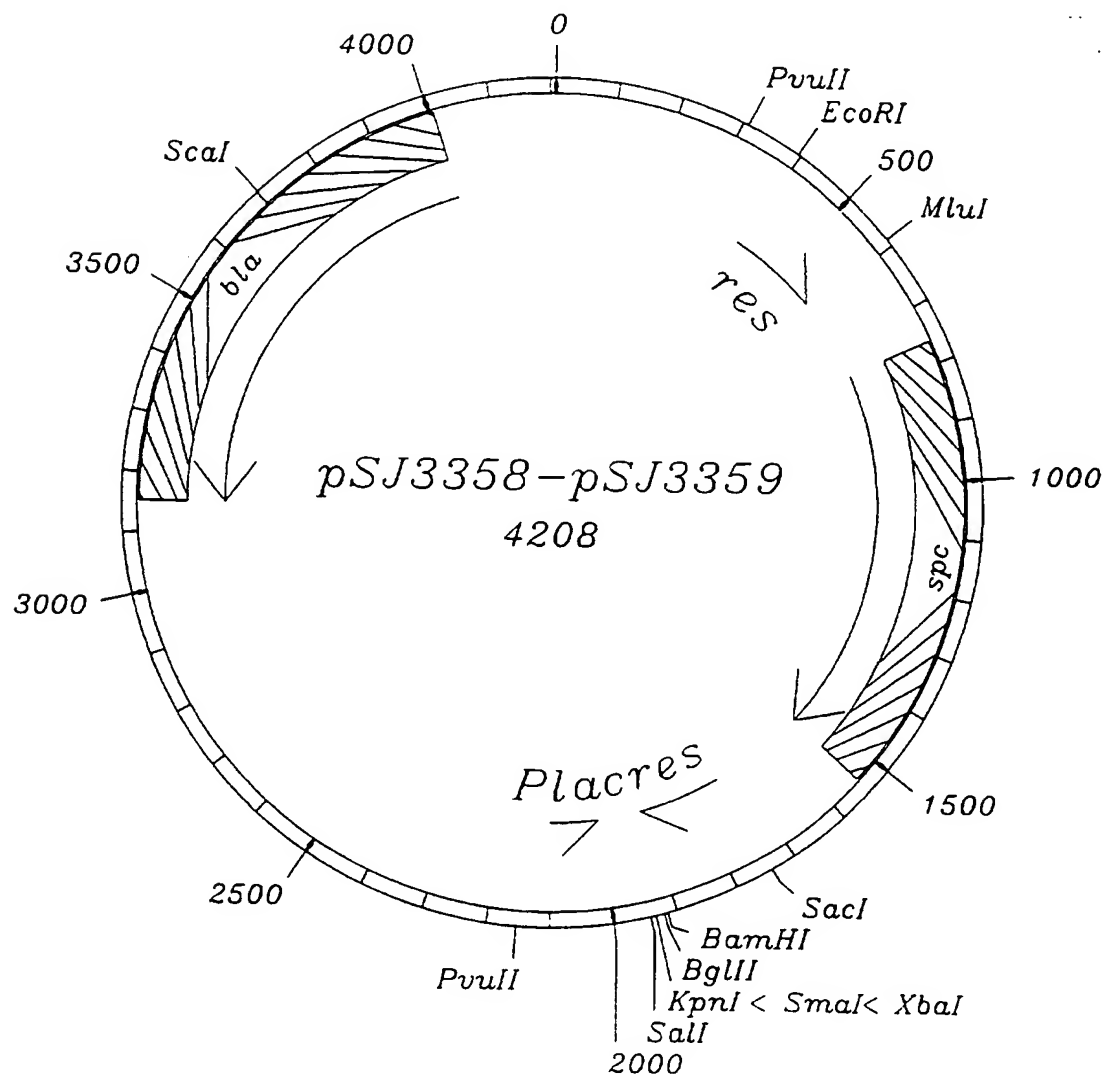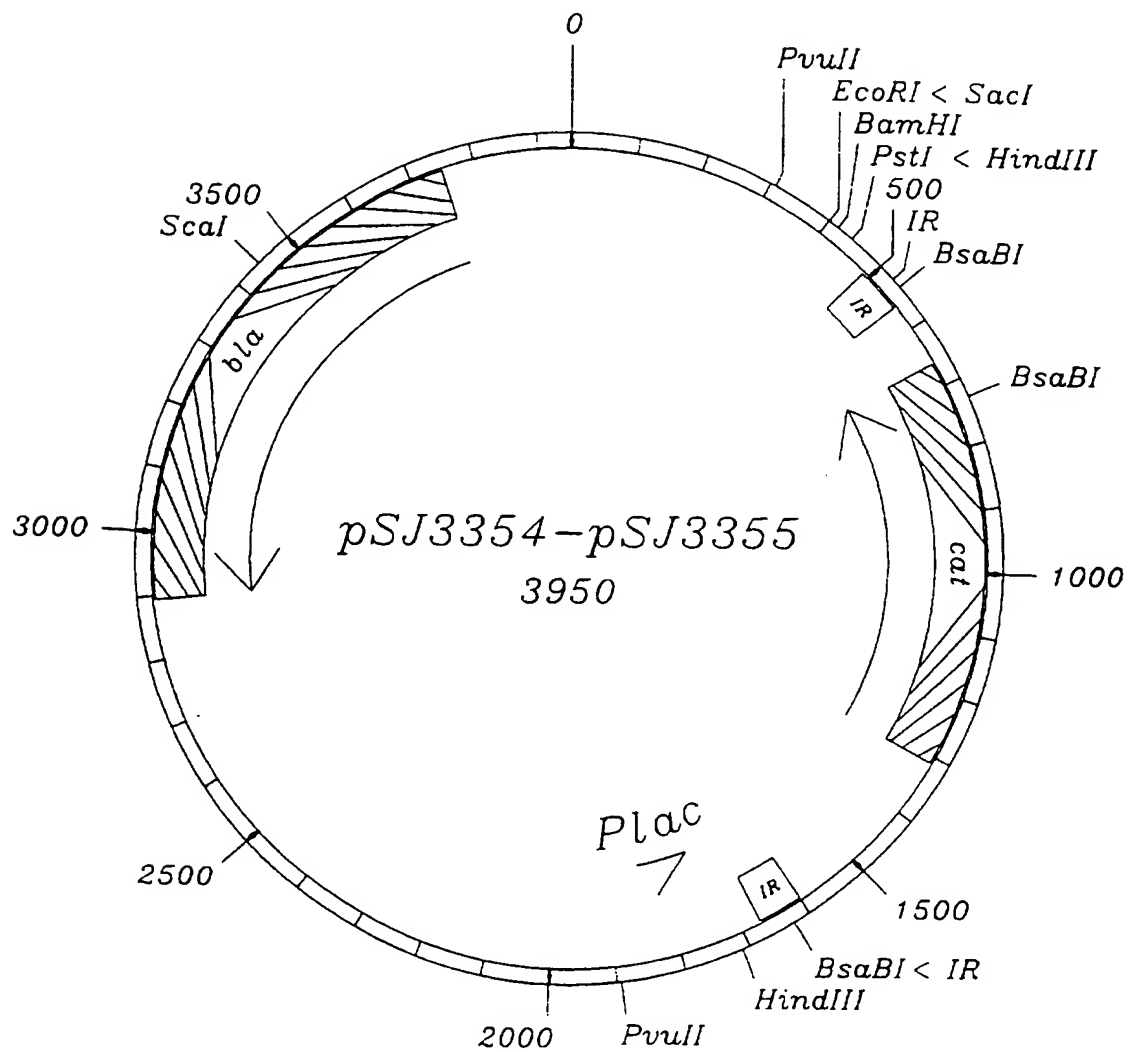
Fig. 13

Fig. 14

Fig. 15

Fig. 16

Fig. 17

Fig. 18

Fig. 19
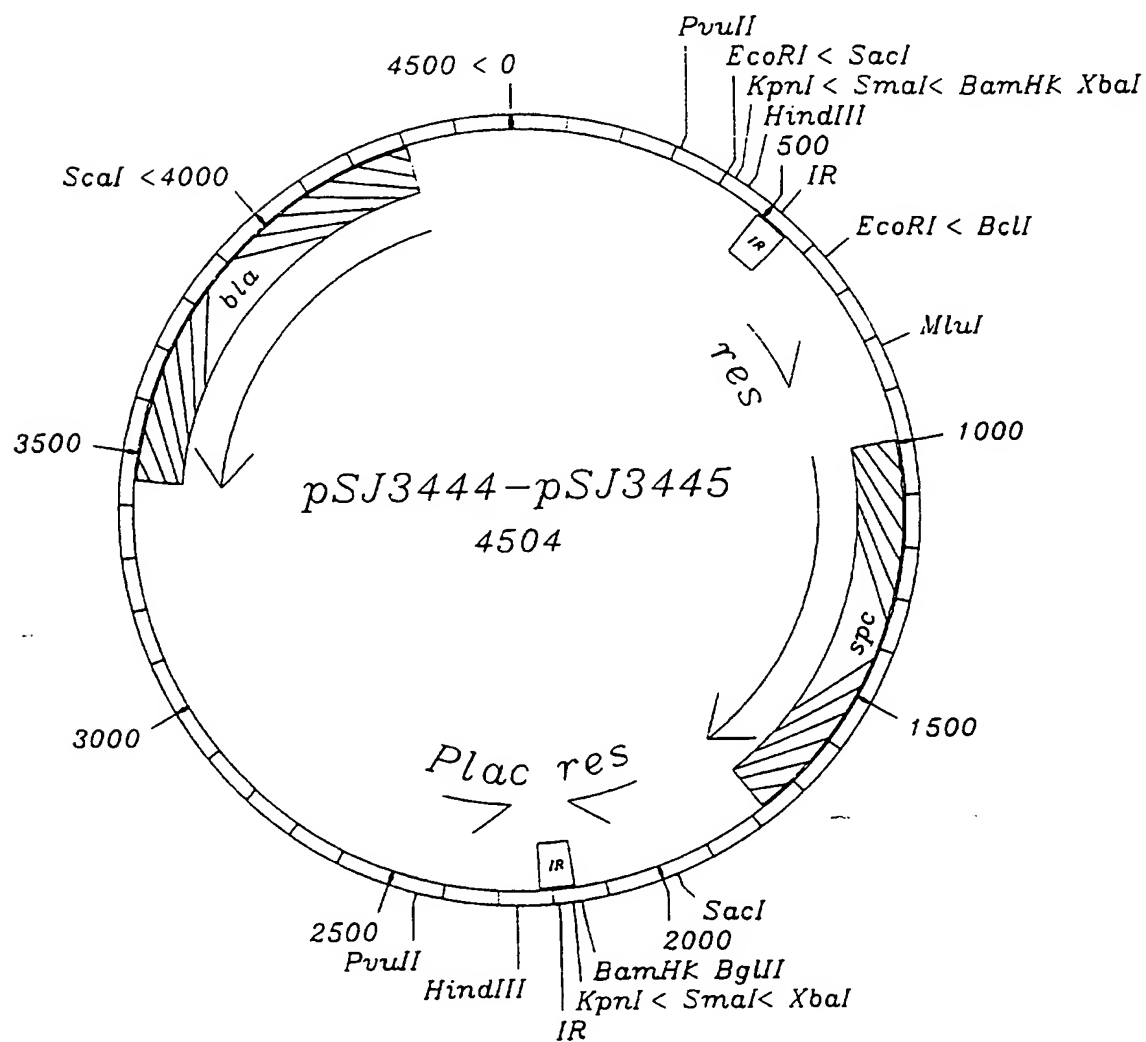
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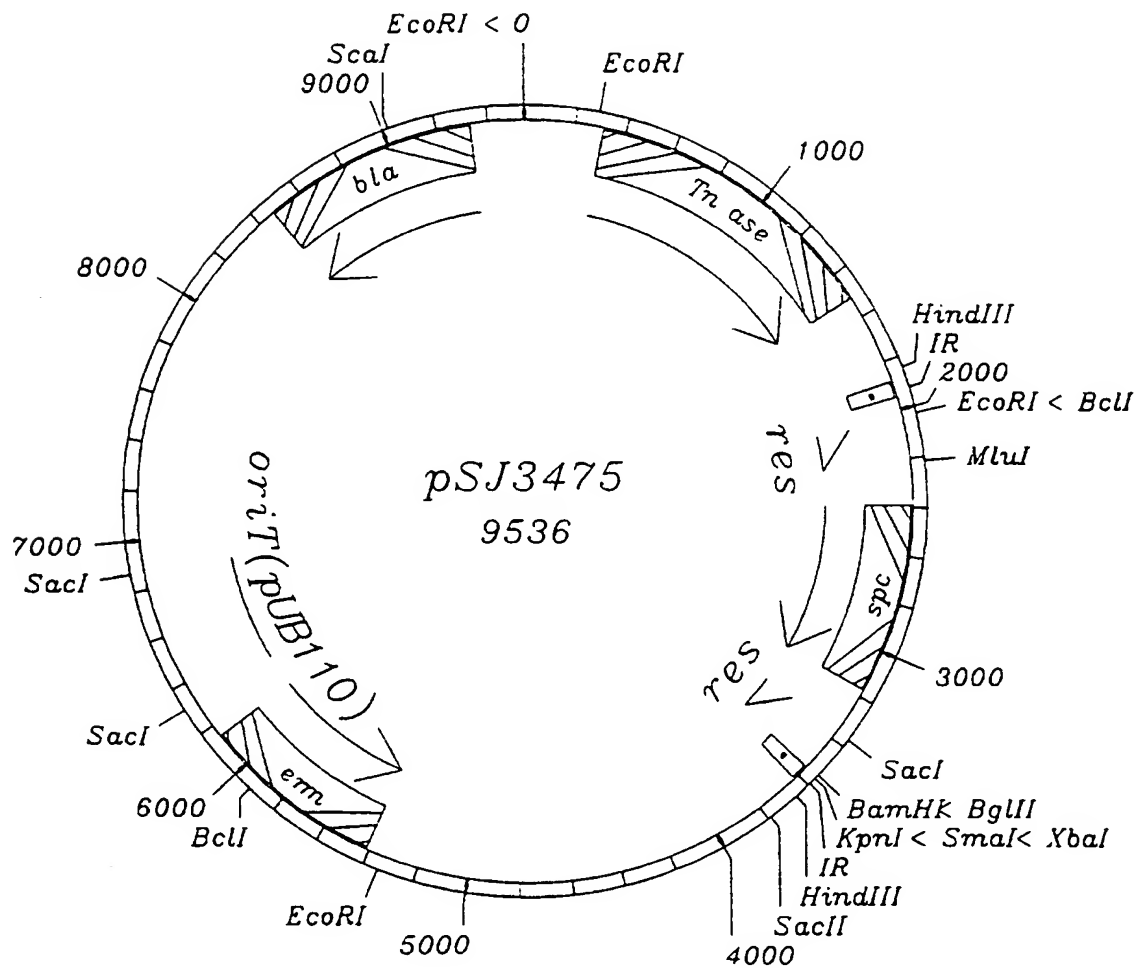
Fig. 21
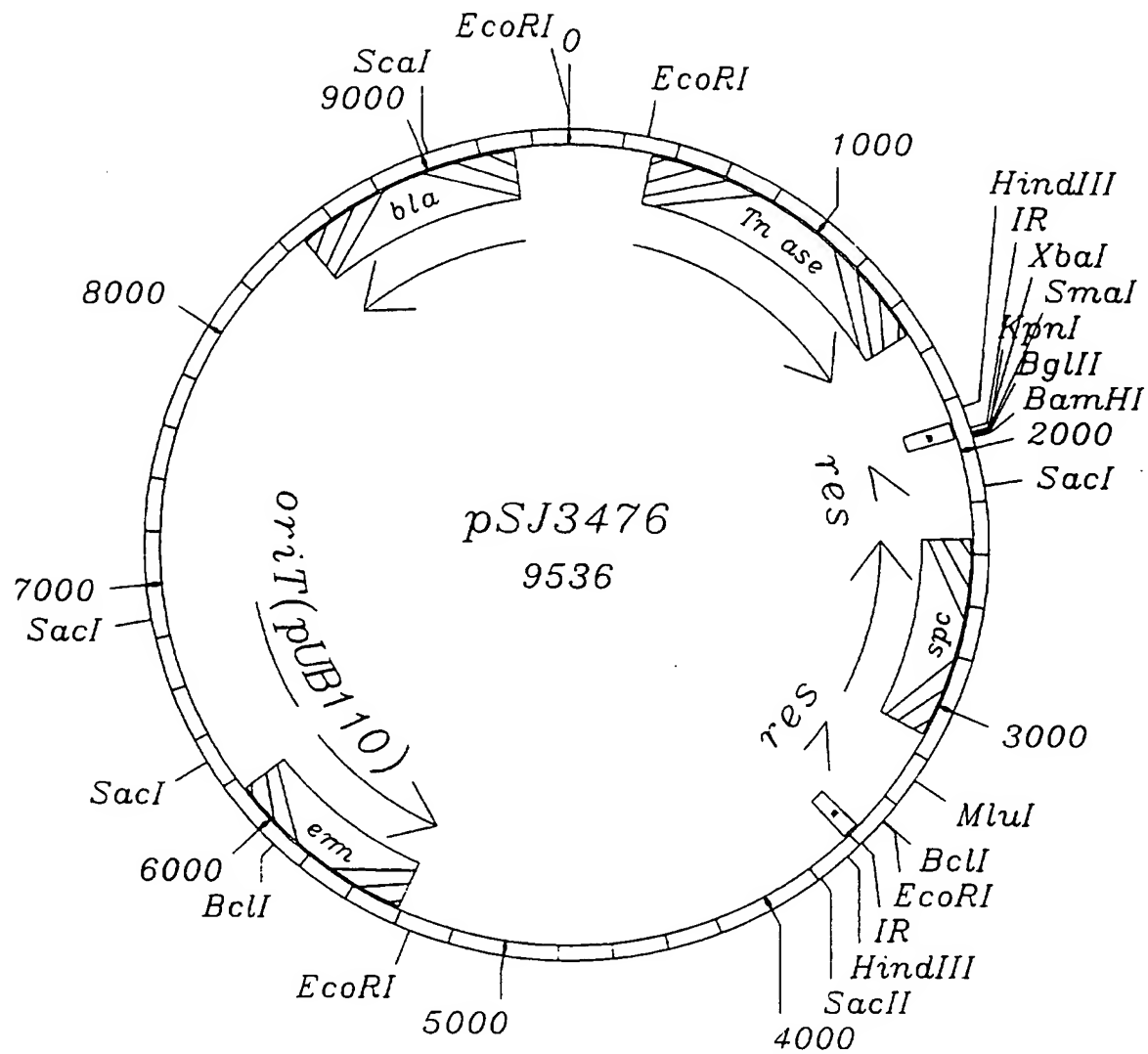
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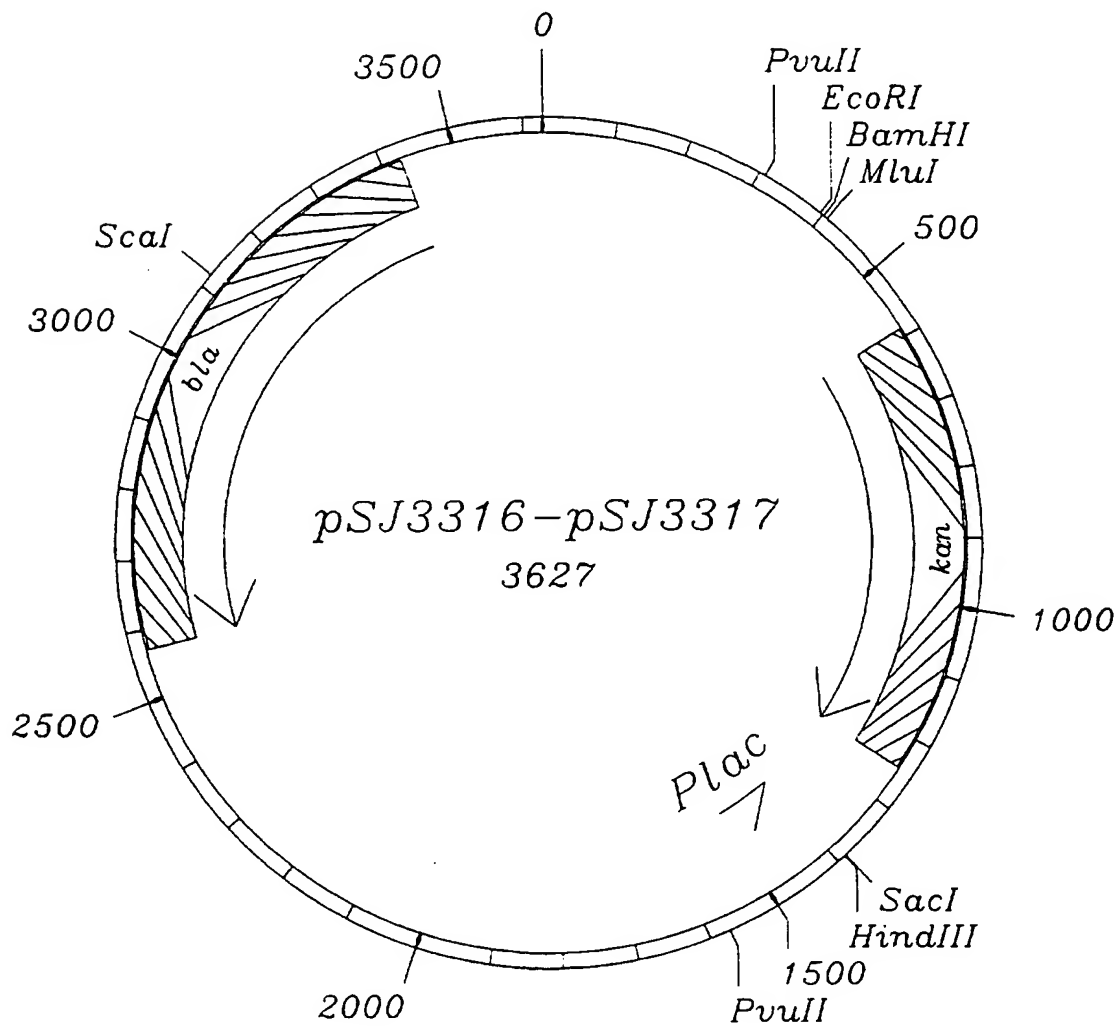
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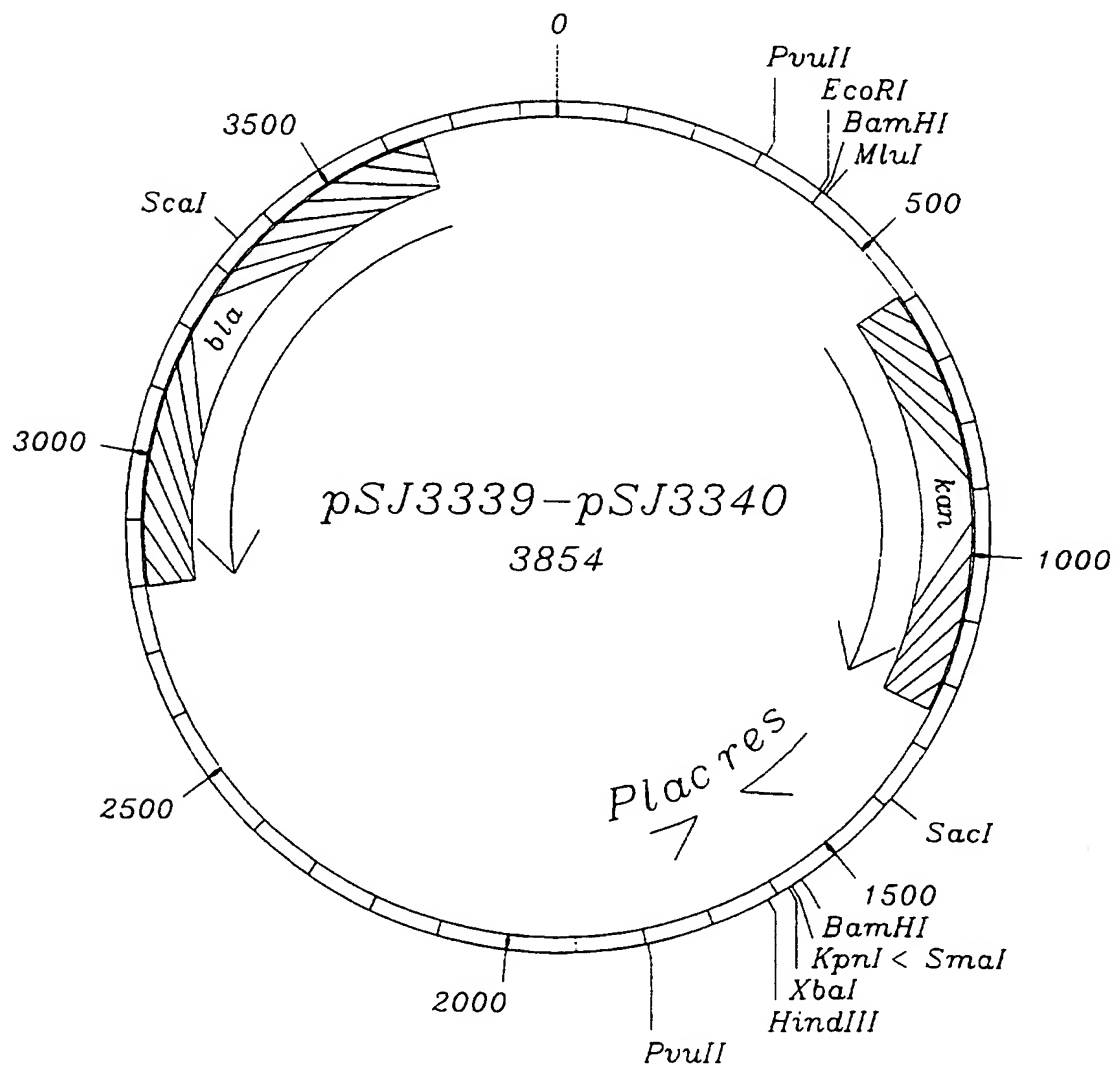
Fig. 24

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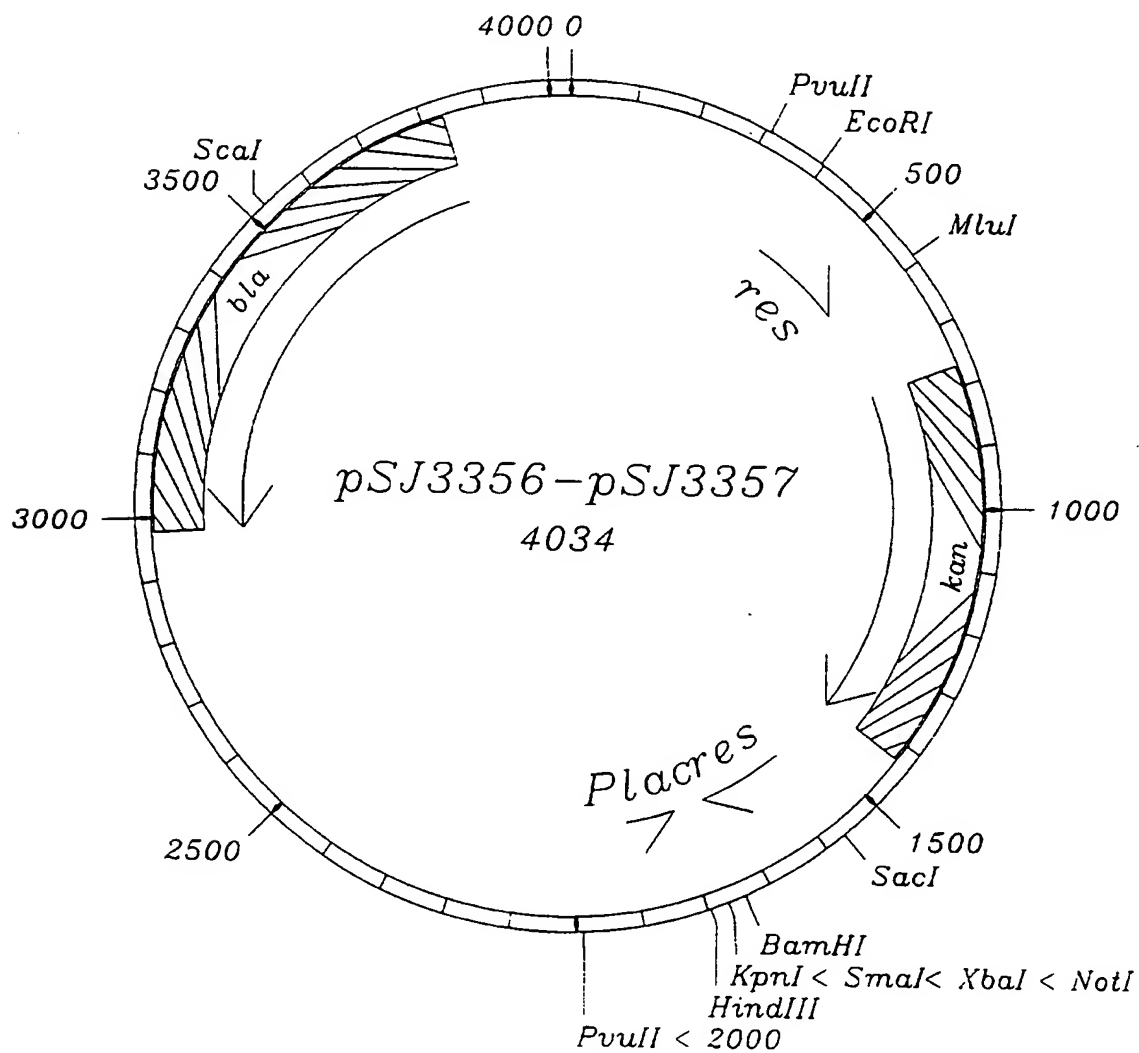
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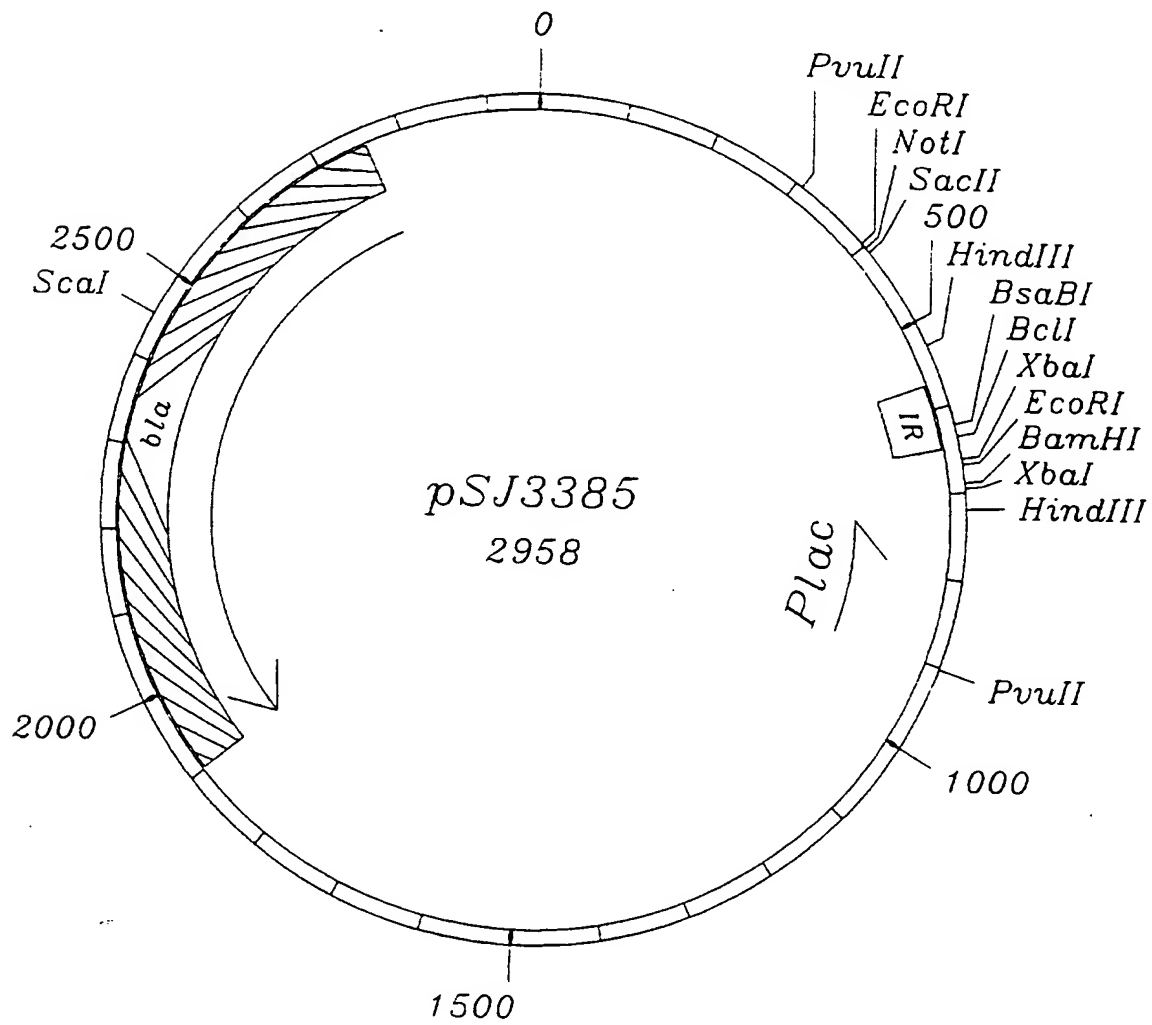
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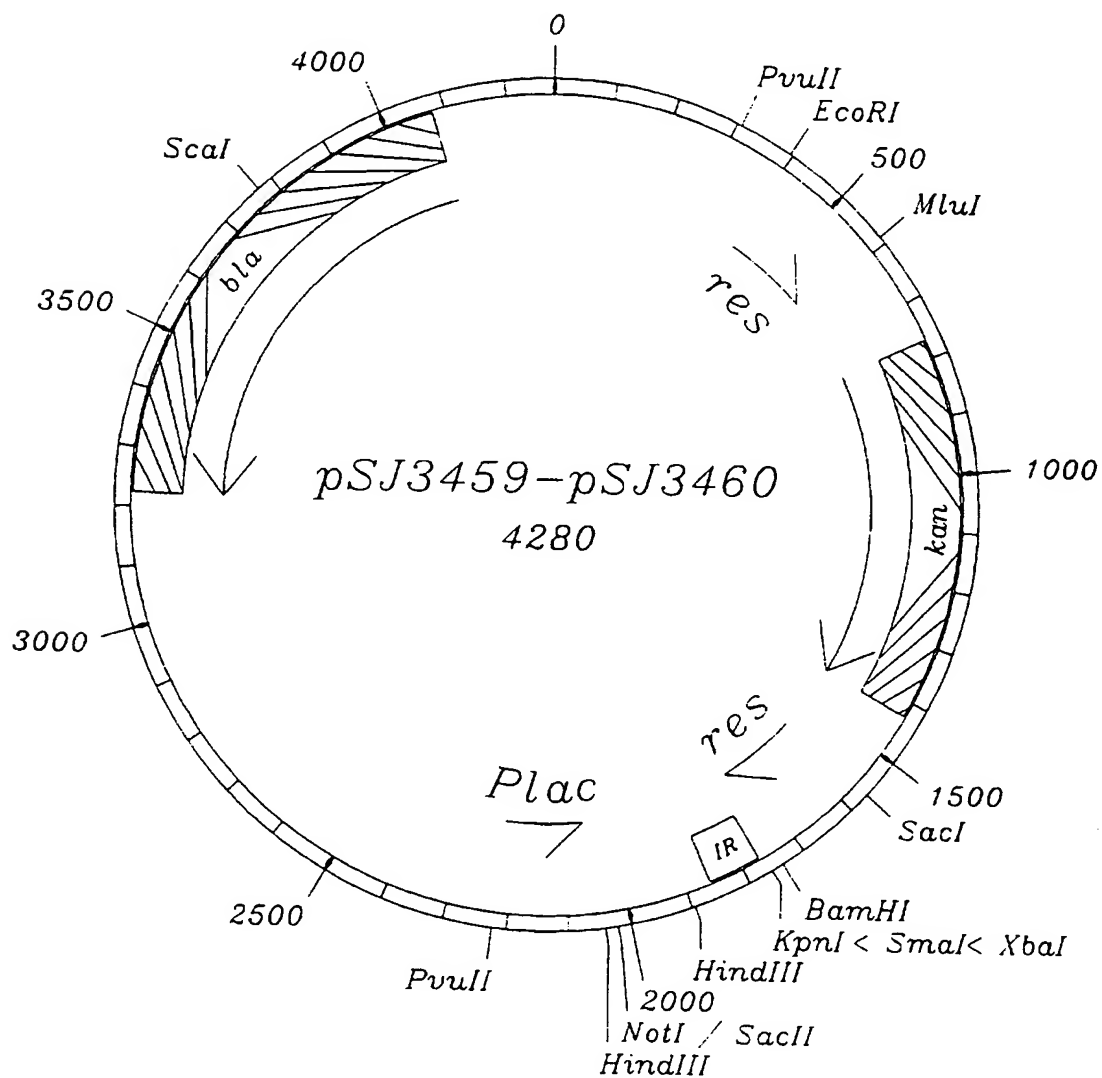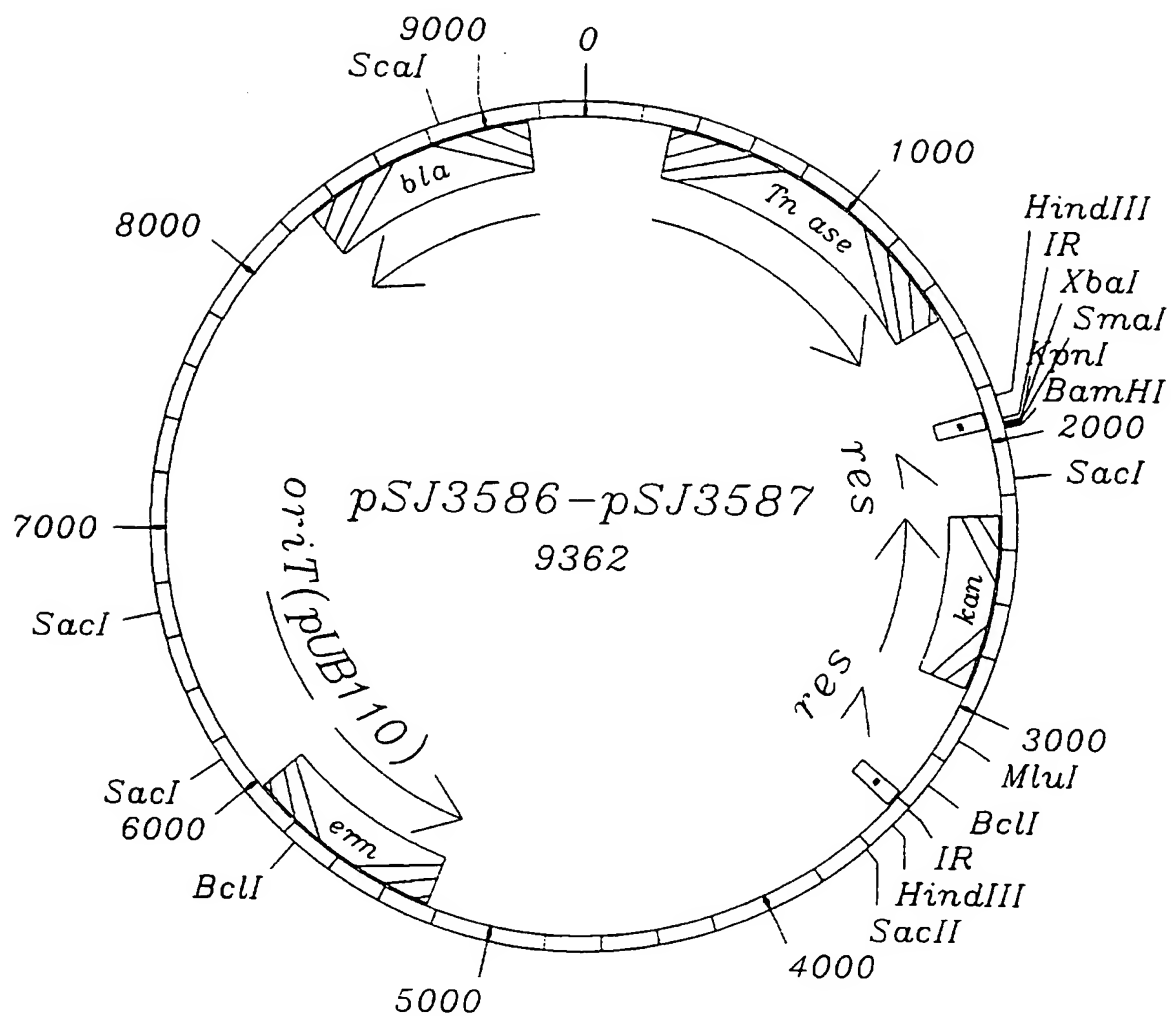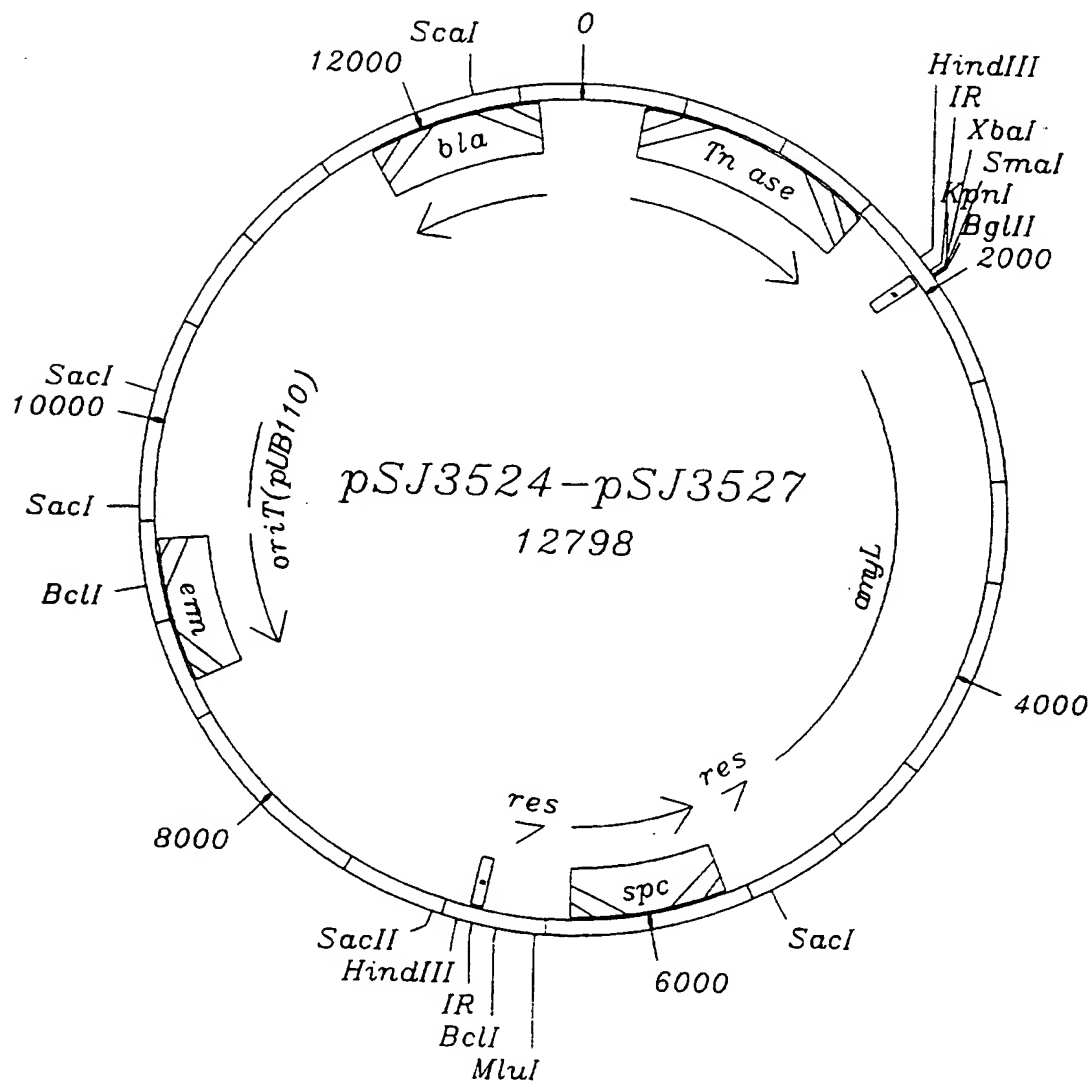
Fig. 27

Fig. 28

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Fig. 29

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 96/00038

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 15/75, C12N 15/65

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPODOC, MEDLINE, BIOSIS, DBA, SCISEAR, PCI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9502058 A2 (ECOGEN INC.), 19 January 1995 (19.01.95), page 7, line 7 - page 9, line 32	1-45
	--	
A	WO 9501095 A1 (BOARD OF SUPERVISORS OF LOUISIANA STATE UNIVERSITY AND AGRICULTURAL AND MECHANICAL COLLEGE), 12 January 1995 (12.01.95), claim 76	1-45
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☐ Further documents are listed in the continuation of Box C.   ☒ See patent family annex.

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"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search

17 April 1996

Date of mailing of the international search report

30 -04- 1996

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Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO-A2-	9502058	19/01/95	NONE	
WO-A1-	9501095	12/01/95	NONE	